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(71) Applicant (for all designated States except US): **INVIT-ROGEN CORPORATION** [US/US]; 1600 Faraday Avenue, Carlsbad, CA 92008 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **MADDEN, Knut, R.** [US/US]; 2705 Glasgow Drive, Carlsbad, CA 92008 (US). **HARRIS, Adam, N.** [US/US]; 5040 Codorniz Way, Apt. 32, Oceanside, CA 92057 (US). **HECKER, Karl, H.** [DE/US]; 10905 Corte Luz Del Sol, San Diego, CA 92130 (US). **LEE, Byung-In** [KR/US]; 550 Verbena Court, Encinitas, CA 92024 (US).

(74) Agents: **ESMOND, Robert, W.** et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., 1100 New York Avenue, N.W., Washington, DC 20005 (US).

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(54) Title: COMPOSITIONS AND METHODS FOR PREPARING SHORT RNA MOLECULES AND OTHER NUCLEIC ACIDS

(57) Abstract: The invention provides methods of preparing nucleic acids, such as RNA molecules, of a defined size or range of sizes. The invention provides compositions, methods and kits for use in the production and preparation of small RNA molecules (including without limitation micro-RNA, siRNA, d-siRNA and e-siRNA) and other nucleic acids of various sizes.

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# COMPOSITIONS AND METHODS FOR PREPARING SHORT RNA MOLECULES AND OTHER NUCLEIC ACIDS

## BACKGROUND OF THE INVENTION

### Field of the Invention

[0001] The present invention is related to the fields of molecular biology, developmental biology, biochemistry and medicine. The invention provides methods of preparing nucleic acids, such as RNA molecules, of a defined size or range of sizes. More specifically, the invention provides compositions and methods for use in the preparation of small RNA molecules and other nucleic acids of various sizes. The invention also provides kits comprising solutions and compositions for preparing Short RNA molecules or other nucleic acids. Further provided are devices and methods for high throughput screening of nucleic acids.

### Related Art

[0002] This summary is not meant to be complete but is provided only for understanding of the invention that follows. The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

### Nucleic Acid Purification

[0003] Methods of purifying nucleic acids are known in the art. Such methods typically involve separating a nucleic acid of interest from other nucleic acids. The separation process is based on differences in parameters such as topology (*e.g.*, supercoiled DNA separated from linear DNA), length (in nucleotides or base pairs for, respectively, single-stranded or double-

stranded nucleic acids), chemical differences, and the like. Although size differences have been used to separate nucleic acids in gels, the methods involved in recovering the separated material in solution phase are time-consuming, as the portion of the gel containing the nucleic acid of interest must be extracted and then treated to degrade the gel or otherwise extract the nucleic acid therefrom, and introduce contaminants from the gel. Such methods are also not easily adapted to high throughput (HTS) screening. The separation of small nucleic acids in solution presents other difficulties.

- [0004]       Methods of purifying certain types of ribonucleic acid (RNA) molecules are known in the art. Such methods include those described in the following publications.
- [0005]       Methods of purifying mRNA from cellular extracts are known in the art. *See, e.g.*, Chapter 7, "Extraction, Purification and Analysis of mRNA from Eukaryotic Cells" in: *Molecular Cloning: A Laboratory Manual*. Sambrook *et al.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001.
- [0006]       Methods of purifying RNA produced by *in vitro* transcription are described by Clarke in "Labeling and Purification of RNA Synthesized by In Vitro Transcription," in: *RNA-Protein Interaction Protocols*. Haynes, ed. Humana Press, Totowa, NJ, 1999.
- [0007]       Puttaraju *et al.*, *Nucleic Acids Symp Ser.* 33:49-51 (1995) describe the purification of circular RNA molecules generated by a modified self-splicing intron-exon sequence.
- [0008]       McLaughlin *et al.*, *J Chromatogr.* 418:51-72 (1987) describe the separation of complex mixtures of tRNAs using high-performance liquid chromatography.
- [0009]       Mandell *et al.*, *Anal Biochem* 1:66 (1960) describe "MAK (methylated albumen on kieselguhr) columns" on which DNA sticks irreversibly, and various sized RNA's can be eluted with higher and higher salt concentrations (kieselguhr is diatomaceous earth). Loeser *et al.*, *Biochemistry* 9:2364-6 (1970) describe the separation of 5S RNA from other nucleic acids by

polyamino acid kieselguhr column chromatography. Modak *et al.*, *Anal Biochem.* 34:284-6 (1970) describe a MAK column procedure for separation of RNA subfractions.

- [0010] These and other methods of nucleic acid isolation are tedious and not readily adaptable to certain applications, such as high throughput screening (HTS) and the purification of small nucleic acids, such as Short RNA (as defined herein). The present invention fulfills this need by providing methods, compositions and kits for the preparation of small nucleic acids. The methods and compositions may also be adapted for use in HTS applications.

#### RNA Interference

- [0011] One example of a methodology that would benefit from methods of preparing relatively pure small nucleic acids is RNA interference (RNAi). RNAi was originally described as a naturally-occurring process in the model organism *C. elegans* (Fire *et al.*, *Nature* 391:806-811, 1998). In brief, the process involves application of double stranded RNA (dsRNA) that represents a complementary sense and antisense strand of a portion of a target gene within the region that encodes mRNA, with the result being post-transcriptional down-regulation of the target gene.

- [0012] Initially, RNAi technology did not appear to be readily applicable to mammalian systems. This is because, in mammals, dsRNA activates dsRNA-activated protein kinase (PKR) resulting in an apoptotic cascade and cell death (Der *et al.*, *Proc. Natl. Acad. Sci. USA* 94:3279-3283, 1997). In addition, it has long been known that dsRNA activates the interferon cascade in mammalian cells, which can also lead to altered cell physiology (Colby *et al.*, *Annu. Rev. Microbiol.* 25:333, 1971; Kleinschmidt *et al.*, *Annu. Rev. Biochem.* 41:517, 1972; Lampson *et al.*, *Proc. Natl. Acad. Sci. USA* 58L782, 1967; Lomniczi *et al.*, *J. Gen. Virol.* 8:55, 1970; and Younger *et al.*, *J. Bacteriol.* 92:862, 1966). However, dsRNA-mediated activation of the PKR and interferon cascades requires dsRNA longer than about 30 base pairs. In contrast, dsRNA less than 30 base pairs in length has been demonstrated to cause RNAi in mammalian



cells (Caplen *et al.*, *Proc. Natl. Acad. Sci. USA* 98:9742-9747, 2001). Thus, it is expected that undesirable, non-specific effects associated with longer dsRNA molecules can be avoided by preparing Short RNA that is substantially free from longer dsRNAs.

[0013] The biochemistry of RNAi involves generation of active small interfering RNA (siRNA) through the action of a ribonuclease, DICER, which digests long double stranded RNA molecules (dsRNA) into shorter fragments. The small interfering RNAs (siRNAs) produced through the action of DICER mediate degradation of the complementary homologous RNA. Since the primary products of DICER are 21-23 base pair fragments of dsRNA, one can circumvent the adverse or undesired mammalian responses to dsRNA and still elicit an interfering RNA effect via siRNA (Elbashir *et al.*, *Nature* 411:494-498, 2001) if the "DICING" reaction goes to completion. Incomplete "DICING," however, results in a mixture of longer RNA molecules, which may trigger undesirable and/or non-specific responses, along with the desired 21-23 bp RNA (*i.e.*, diced siRNA or d-siRNA) molecules. It is thus desirable to separate Short RNA of the desired narrow size range (from about 21 to about 23) from other dsRNA (*e.g.*, dsRNA substrate, incompletely "diced" dsRNA, contaminating RNA, and the like) in order to prepare d-siRNA compositions having a higher specific RNAi activity.

[0014] Another enzyme that has been used to catalyze the *in vitro* processing of long dsRNA substrates to shorter siRNA molecules is RNase III, particularly prokaryotic RNase III, *e.g.*, *Escherichia coli* RNase III (Yang *et al.*, *Proc Natl Acad Sci USA* 99: 9942-7, 2002; Calegari *et al.*, *Proc Natl Acad Sci USA* 99:14236-40, 2002). Complete digestion of dsRNA with RNase III results in Short RNA averaging from about 12 to about 15 bp in length, but these short dsRNA molecules have been reported to not be as effective at triggering an RNAi response in mammalian cells (Paddison *et al.*, *Proc. Natl. Acad. Sci. USA* 99:1443-8, 2002). Limited RNase III digestion of dsRNA is used to obtain Short RNA having a length of from about 20 to about 25 bp. These Short RNA molecules, which have been called endoribonuclease-

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[0017] In some embodiments of the invention, the siRNA that is desired to be prepared has 3' overhangs having from about 1 to about 5 nt. In these and other embodiments, the siRNA comprises a core having from about 10 to about 30 bp; from about 15 to about 25 bp; or 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29 bp.

[0018] There is a need for methods, compositions and kits by which one can prepare nucleic acids, particularly small nucleic acids, more particularly Short RNA (as defined herein) including without limitation micro-RNA, siRNA, d-siRNA and e-siRNA. There is also a need for devices (*e.g.*, filter blocks) comprising such compositions that can be used for high throughput screening of nucleic acids, particularly small nucleic acids, more particularly Short RNA molecules.

## SUMMARY OF THE INVENTION

[0019] The present invention provides methods and compositions for preparing one or more Short RNA molecules or other nucleic acids. Short RNA is used herein as a non-limiting example of a nucleic acid to which the invention can be applied; other nucleic acids are nonetheless within the scope of the invention.

[0020] The term "preparing one or more nucleic acids" is meant to encompass methods in which a composition comprising a population of nucleic acids is treated in order to produce a composition comprising a subpopulation of nucleic acids. In general, the subpopulation is defined by differences in size among the population of nucleic acids, although other characteristics may also be of interest. Other characteristics include, by way of non-limiting example, the chemical structure of the nucleic acid (*i.e.*, the chemical differences between DNA, RNA and PNA, as well as chemical modifications of nucleic acids); secondary structure (*e.g.*, stem-loop sequences, double-strandedness vs. single-strandedness, circular vs. linear); topology (supercoiled vs. relaxed); and the like.

[0021] As an example of the meaning of the term “preparing a nucleic acid”, in the phrase “preparing Short RNA”, the term “preparing” includes but is not limited to (i) fractionating a sample to produce fractions thereof that comprise a given size or range of sizes of RNA molecules, (ii) enriching for RNA molecules of a given size or range of sizes, (iii) separating RNA molecules of a given size or range of sizes from other components of a biochemical reaction (e.g., enzymes, buffer components such as salts, cofactors and/or unreacted substrates, including by way of non-limiting example unreacted nucleic acids), (iv) purifying one or more Short RNA molecules of a given size or range of sizes, and (v) isolating RNA molecules of a given size or range of sizes.

[0022] Four non-limiting exemplary modalities of the methods of the invention are a “first 1 column” method, a “second 1 column” method, a “2 column” method, and an alcohol gradient fractionation method. These are described in the following sections in the context of purifying short (21-23 bp) double-stranded siRNA molecules (d-siRNA) from a reaction in which a longer template dsRNA has been treated with an enzyme (an RNase) that cleaves the template dsRNA into the short d-siRNA molecules. The RNase is selected from the group consisting of ribonuclease A, nuclease S1, ribonuclease T1, RNase III, and DICER.

[0023] In particular embodiments of the invention, the RNase is DICER. In these exemplary embodiments, the desired Short siRNA (d-siRNA) has a length of from about 16 bp to about 30 bp, preferably from about 20 to about 25 bp, and most preferably from 21 bp to 23 bp. The desired Short d-siRNA is desirably separated from (i) the DICER enzyme, (ii) reaction mix components (salts, triphosphates, etc.), (iii) non-nucleic acid products resulting from digestion of the RNA (bases, sugars, etc.), (iv) unreacted (template) dsRNA, and (v) partially reacted dsRNA. The last of these undesirable components is often the most difficult to separate from the desired 21-23 bp d-siRNA, as they can be as small as about 30 bp in length.

[0024] In particular embodiments of the invention, the RNase is a member of the RNase III family of ribonucleases (Lamontagne *et al.*, *Curr Issues Mol*

*Biol.* 3:71-78, 2001), including without limitation a prokaryotic RNase III (e.g., RNase III from *E. coli*). In these exemplary embodiments, the desired Short RNA (e-siRNA) has a length of from about 15 or 16 bp to about 30 bp, preferably from about 18 bp to about 28 bp, and most preferably from 20 to 25 bp. The desired Short e-siRNA is desirably separated from (i) the RNase III enzyme, (ii) reaction mix components (salts, triphosphates, etc.), (iii) non-nucleic acid products resulting from digestion of the RNA (bases, sugars, etc.), (iv) unreacted (template) dsRNA, (v) partially reacted dsRNA roughly equal to or greater than about 30 bp in length, and (vi) over-digested RNA products equal to or less than about 18 bp. The last two of these undesirable components is often the most difficult to separate from the desired 21-23 bp d-siRNA.

[0025] In a first 1-column modality, methods of preparing a Short RNA of a preselected size comprise:

[0026] (a) adding a fluid mixture or, in any order, fluids or combinations of fluids that contain the components of said fluid mixture, to a sample comprising Short RNA molecules and other nucleic acids to produce a binding mixture;

[0027] (b) filtering the binding mixture through an affinity column, wherein Short RNA molecules and other nucleic acids bind to a composition within the affinity column; and optionally washing the bound Short RNA and other nucleic acid molecules; and

[0028] (c) eluting the bound Short RNA molecules with a second fluid mixture,

wherein Short RNA molecules are present in the eluate, and other nucleic acids remain bound to the composition within the affinity column.

[0029] In these and other embodiments, particularly kit-related embodiments for the purification of RNA, a solution comprising all the components of a fluid mixture except an alcohol may be referred to as an "RNA Binding Buffer", and the solution used to optionally wash bound Short RNA may be called an "RNA Wash Buffer". The RNA Binding Buffer may comprise from about 1

to about 9 M, 1 M, 2 M, 3 M, 4 M, 5 M, 6 M, 7 M, 8 M or 9 M guanidine isothiocyanate. For example, a preferred RNA Binding Buffer is 4 M guanidine isothiocyanate, 50 mM Tris-HCl, pH 7.5, 25 mM EDTA, pH 8.0, and, optionally, 1%  $\beta$ -mercaptoethanol. This RNA Binding Buffer is mixed 1:1 with 100% ethanol to prepare a fluid mixture according to the invention. A fluid mixture prepared in this fashion can be from about 1 to about 2 M, 1 M, 1.1 M, 1.15 M, 1.2 M, 1.25 M, 1.3 M, 1.31 M, 1.32 M, 1.33 M, 1.34 M, 1.35 M, 1.4 M, 1.45 M, 1.5 M, 1.55 M, 1.6 M, 1.65 M, 1.7 M, 1.75 M, 1.8 M, 1.85 M, 1.9 M, 1.95 M or 2 M guanidine isothiocyanate. A preferred RNA Wash Buffer is 5 mM Tris-HCl (pH 7.5), 0.1 mM EDTA (pH 8.0), and 80% ethanol. In general, in this and other solutions and buffers of the invention, EDTA can be substituted for by another chelating agent, preferably a divalent cation chelator, such as EGTA and the like.

[0030] In a second 1-column modality, the methods of the invention comprise:

[0031] (a) adding a fluid mixture or, in any order, fluids or combinations of fluids that contain the components of said fluid mixture, to a sample comprising Short RNA molecules and dsRNA to produce a binding mixture;

[0032] (b) filtering the binding mixture through an affinity column, wherein Short RNA molecules pass through the column, and wherein dsRNA having at least about 25% more base pairs than the Short RNA is retained in the column.

[0033] In further embodiments, the dsRNA has at least about 30%, 40%, 50%, 60%, 70%, 80%, 95%, two-fold, three-fold, four-fold, five-fold, ten-fold, twenty-fold, fifty-fold, or a hundred-fold more base pairs than the Short RNA.

[0034] The 1-column modalities are particularly useful for the purification of nucleic acids prepared by *in vitro* chemical synthesis. Generally, in such syntheses, two oligonucleotides are prepared in separate syntheses and are hybridized (annealed) to each other to generate a Short dsRNA molecule. This hybridization mixture is a non-limiting example of a sample comprising Short RNA, and the undesirable partial products that are preferably removed therefrom include unincorporated nucleotides and unhybridized

oligonucleotides. Other contaminants (chemical contaminants from the synthesis reactions, salts in hybridization buffers, and the like) are also preferably removed by the methods of the invention.

[0035] Optionally, the method further comprises (d) precipitating the RNA in the eluate with an alcohol in the presence of a coprecipitant, such as yeast tRNA and other nucleic acids, glycogen, and the like. Because it does not contain nucleic acids, glycogen does not result in contamination of the nucleic acid that is desirably purified (*e.g.*, siRNA) with other nucleic acids. Typically, the precipitation in (d) involves the addition of an alcohol at temperature of from about 0°C to about 40°C, placing the mixture on ice for about 10 min, and applying centrifugal force by spinning the mixture (in a microfuge in instances where volumes less than about 2 ml are used) for 30 min. The precipitated RNA can be dried and stored or can be resuspended in a buffer.

[0036] In various embodiments, the fluid mixture comprises an alcohol, such as ethanol or isopropanol. In such embodiments, the first fluid mixture comprises between from about 1% to about 50% of an alcohol by volume (*i.e.*, v/v). By "between from about 1% to about 50% alcohol" it is meant that the solution is comprised of about s% of an alcohol (*e.g.*, ethanol or isopropanol), wherein "s" is any integer between 1 and 50, including without limitation 33%, particularly about 33% ethanol.

[0037] In some embodiments, the preparative methods of the present invention utilize affinity columns that comprise one or more glass fiber segments, but other types of affinity columns can be used.

[0038] In 2-column modalities, the methods of the invention comprise:

[0039] (a) adding a first fluid mixture or, in any order, fluids or combinations of fluids that contain the components of said first fluid mixture, to a sample comprising Short RNA molecules of a preselected size, to produce a first binding mixture;

[0040] (b) filtering the first binding mixture through a first affinity column, wherein Short RNA molecules pass through a composition within the first

affinity column, and RNA molecules having a size that is at least twofold greater than that of said preselected size are retained, to produce a first flow-through solution;

[0041] (c) adding a second fluid mixture or, in any order, fluids or combinations of fluids that contain the components of said second fluid mixture, to the flow-through solution, to produce a second binding mixture;

[0042] (d) filtering the second binding mixture through a second affinity column, wherein Short RNA molecules bind to a composition within the second affinity column and, optionally, washing the bound Short RNA molecules; and

[0043] (e) eluting the bound Short RNA molecules with a third fluid mixture, or, in any order, fluids or combinations of fluids that contain the components of said third mixture, wherein Short RNA molecules are present in the eluate.

[0044] One or more of the affinity columns can, but need not, comprise one or more glass fiber segments, and other types of affinity columns can be used.

[0045] The 2-column modality is particularly useful for the purification of nucleic acids prepared by RNase digestion *in vitro*. In general, a long dsRNA molecule is prepared by methods known in the art, and is treated with RNase in order to generate a reaction mixture comprising, among other things, Short dsRNA molecules. This reaction mixture is a non-limiting example of a sample comprising Short RNA, and the undesirable partial products that are preferably removed therefrom include unincorporated nucleotides and unhybridized oligonucleotides.

[0046] As a non-limiting example, a substrate dsRNA that is about 500 bp long is separated from diced Short RNA molecules. As another example, the method is used to separate a completely diced RNA fragment (*e.g.*, a 22-mer) from incompletely diced Short RNA molecules (*e.g.*, 44-mers, 66-mers, 88-mers, etc.). Small molecules (*e.g.*, nucleotides removed from RNA molecules by DICER, salts, ions, nucleotides and mono-, di- and tri-phosphates thereof) and proteins (enzymes, *e.g.*, ribonucleases), and components of enzyme "stop solutions" can also be separated from the desired Short RNA molecules. A



"stop solution" for DICER or another RNase may comprise EDTA, EGTA or some other chelating agent, KCl, and/or an RNase inhibitor (such as RNasin® Ribonuclease Inhibitor from Promega, Madison, WI).

[0047]        Optionally, the method further comprises (f) precipitating the RNA in the eluate with an alcohol with a coprecipitant such as, for example, glycogen, which is, as explained above, preferred in some instances and embodiments. The precipitated RNA can be dried and stored or resuspended in a buffer.

[0048]        In this and various other embodiments of the invention, the first and second affinity columns are identical; in other embodiments, they are different.

[0049]        In modalities involving alcohol gradient fractionation, the methods of the invention comprise:

[0050]        (a) adding a first fluid mixture to a nuclease reaction mix, to produce a first binding mixture, wherein the first fluid mixture is essentially free of alcohol;

[0051]        (b) filtering the first binding mixture through a first affinity column, wherein longer RNA molecules in the first binding mixture (*i.e.*, RNA having a length of about "y" bp to about "z" or more bp) bind to, and shorter RNA molecules (*i.e.*, RNA having a length of about 5 bp to about "y" bp) pass through, a composition within the first affinity column, to produce a first flow-through solution comprising RNA having a length of about 5 bp to about "y" bp;

[0052]        (c) eluting the longer RNA from the first affinity column, to produce a first eluate comprising longer RNA molecules having lengths in the range of about "y" bp to about "z" or more bp;

[0053]        (d) adding one or more alcohol(s) to the first flow-through solution to bring the final concentration to about 10% (v/v), to produce a second binding mixture;

[0054]        (e) filtering the second binding mixture through a second affinity column, wherein longer RNA molecules in the second binding mixture (*i.e.*, RNA having a length of about "x" bp to about "y" or more bp) bind to, and

shorter RNA molecules (*i.e.*, RNA having a length of about 5 bp to about “x” bp) pass through, a composition within the second affinity column, to produce a second flow-through solution comprising RNA having a length of about 5 bp to about “x” bp;

[0055] (f) eluting the longer RNA from the second affinity column, to produce a second eluate comprising longer RNA molecules having lengths in the range of about “x” bp to about “y” bp;

[0056] (g) adding one or more alcohol(s) to the second flow-through solution to bring the final concentration to about 10% (v/v) more than the first flow-through solution, to produce a third binding mixture;

[0057] (h) filtering the third binding mixture through a third affinity column, wherein longer RNA molecules in the second binding mixture (*i.e.*, RNA having a length of about “w” bp to about “x” bp) bind to, and shorter RNA molecules (*i.e.*, RNA having a length of about 5 bp to about “w” bp) pass through, a composition within the third affinity column, to produce a third flow-through solution comprising RNA having a length of about 5 bp to about “w” bp; and

[0058] (i) eluting the longer RNA from the second affinity column, to produce a third eluate comprising longer RNA molecules having lengths in the range of about “w” bp to about “x” bp;  
wherein “w” < “x” < “y” < “z”, and wherein said first eluate comprises RNA molecules having an average length greater than that of the RNA molecules in the second eluate, and the second eluate comprises RNA molecules having an average length greater than that of the RNA molecules in the third eluate.

[0059] In some embodiments, steps (g) through (i) are repeated for as many cycles as is necessary to achieve the desired degree of size-based fractionation. In this modality, the % (v/v) alcohol in each flow-through solution is increased by about 10% until RNA of the desired (short) length is retained by and then eluted from an affinity column. Additionally or alternatively, the alcohol may be added at more discrete concentrations (*e.g.*, about 5%, followed by about 10%, followed by about 15%, etc.) in order to produce a series of eluates

comprising RNA molecules, each eluate having narrower ranges of length (bp) than eluates produced with less discrete concentrations of alcohol.

- [0060] Optionally, the method further comprises precipitating the RNA in the eluate with an alcohol as described above. The precipitated RNA can be dried and stored or can be resuspended in a buffer.
- [0061] In various embodiments of this aspect of the invention, the first and/or second affinity column(s) are glass fiber columns.
- [0062] In various embodiments of this and other aspects of the invention, isopropanol is substituted for ethanol. Other alcohols, and combinations of alcohols, can be used to practice the invention.
- [0063] The methods, compositions and kits of the present invention may be used without an RNA digestion step, that is, simply to separate Short RNA molecules within the size range of about 10 to about 30 nucleotides or base pairs in length from a plurality of Short RNA molecules (or other nucleic acids), as well as proteins (*e.g.*, enzymes, including without limitation RNases) and small organic compounds (*e.g.*, salts and ions; free bases, nucleotides, and mono-, di- and tri-phosphates thereof; and the like). For example, the invention may be used to prepare tRNA from a cellular lysate.
- [0064] The methods, kits and compositions of the invention can be used to separate a monomeric form of a Short RNA molecule from a solution comprising both monomeric and multimeric forms of the Short RNA molecule. For example, an RNase may be a processive enzyme that cuts fragments of short length (*e.g.*, 20-25 nt or bp) from a longer template RNA molecule. In such a situation, if the RNase reaction does not go to completion, a series of incompletely digested template RNA molecules is generated.
- [0065] For example, an RNA template comprising 5 repeats of a Short RNA sequence will yield, if its digestion is incomplete, the desired monomeric form as well as multimeric forms (*e.g.*, dimers, trimers, tetramers and the template RNA, which can be thought of as a "5-mer"). The methods, compositions and kits of the invention are used to separate monomers from the multimers. For example, a Short RNA molecule 21 bp in length is separated from

incompletely digested (multimeric) molecules, *i.e.*, Short RNA molecules having lengths of 42, 63, 84, or 105 bp; a Short RNA 22 bp in length is separated from Short RNA molecules having lengths of 44, 66, 88, or 110 bp; and a Short RNA 23 bp in length is separated from Short RNA molecules having lengths of 46, 69, 92, or 115 bp.

[0066] Once separated from template and incompletely digested RNA molecules, the monomeric form of the Short RNA molecules have enhanced activity. Non-limiting examples of enhanced activity of Short RNA molecules include greater specificity (*i.e.*, the regulation of the target sequences and genes occurs with less background and/or fewer spurious effects); higher specific activity (whereby a lower dose of the purified Short RNA molecule is required to achieve the same result as with a higher dose of unpurified Short RNA molecules); reduced toxicity on the subcellular, cellular and/or organismal level; increased stability *in vitro* or *in vivo*, which may include an enhanced shelf life; and the like. The present invention also provides kits useful for carrying out the methods of the invention.

[0067] Other preferred embodiments of the present invention will be apparent to one of ordinary skill in light of the following drawings and description of the invention, and of the claims.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0068] Figure 1 diagrams the "1 column" modality of the invention Panel A: Schematic of the purification method. Panel B: Gel showing results of purifying Dicer reaction products using the 1 column modality of the invention with different concentrations of ethanol (EtOH) in the RNAbindign buffer. Lane 1: "L", 10-bp ladder, with 20-bp and 30-bp bands noted; lane 2: crude siRNA reaction ("Rxn"); lanes 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12: flowthrough of binding buffer containing 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% and 50% EtOH, respectively. The positions of the dsRNA Dicer substrate and siRNA product are indicated on the right.

- [0069] Figure 2 shows activity and specificity of siRNA fractions. Panel A: measurement of luciferase activity in cells after transfection with lacZ siRNA fractions. Panel B: measurement of beta-gal activities after transfected with lacZ siRNA fractions.
- [0070] Figure 3 shows the application of the "2 column" modality of the invention to siRNA. Panel A: Schematic of purification method. Panel B: a 20% polyacrylamide TBE gel showing (i) 10 bp ladder (leftmost lane); (ii) dsRNA Dicer substrate, unpurified dicing reactions, partially purified and purified d-siRNA molecules for GFP (lanes 2-5); (iii) dsRNA Dicer substrate, unpurified dicing reactions, partially purified and purified d-siRNA molecules for luc (lanes 6-9), and (iv) synthetic siRNA (lane 10).
- [0071] Figure 4 shows the "alcohol gradient fractionation" modality of the invention applied to short dsDNA molecules in a DNA "ladder" (L). Panel A: Schematic of purification method. Ethanol concentrations are systematically increased in flow-through before binding to subsequent columns. Panel B: 20% polyacrylamide TBE gel showing 10 bp ladder (L) and eluates from Micro-to-Midi RNA purification columns bound with the percent ethanol indicated (*i.e.*, 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70% and 80% EtOH).
- [0072] Figure 5 shows a Western analysis for detecting an endogenous gene (lamin A/C) in cells contacted with siRNA targeted to lamin A/C or control siRNA targeted to lacZ.
- [0073] Figure 6 shows a diagram of the iRNA process and pathway.
- [0074] Figure 7 shows an example of expected results of a *lacZ* dicing reaction.
- [0075] Figure 8 shows a flow digram illustrating the d-siRNA purification process.
- [0076] Figure 9 shows an example of expected results following purification of *lacZ* d-siRNA.
- [0077] Figure 10 shows ds-iRNA inhibition of luciferase and  $\beta$ -galactosidase as percent of control versus transfection condition.

- [0078] Figure 11 shows inhibition of expression of lamin A/C expression using d-siRNA.
- [0079] Figure 12 illustrates the major steps necessary to generate dsRNA using the BLOCK-iT™ RNAi TOPO® Transcription System.
- [0080] Figure 13 shows TOPO® linking to a PCR product.
- [0081] Figure 14 shows the RNAi process and pathway.
- [0082] Figure 15 is a diagram of the BLOCK-iT T7-TOPO linker.
- [0083] Figure 16 shows an analysis of an annealing reaction of GFP and luciferase dsRNA samples.
- [0084] Figure 17 is a vector map of pcDNA™1.2/V5-GW/lacZ.
- [0085] Figure 18 shows fractionation of double-stranded RNA using different ethanol concentrations.
- [0086] Figures 19A-19C show: 19A) gel analysis results of crude lacZ siRNA, siRNA purified using the two-column protocol, various fractions of the single-column purification protocol, as well as chemically synthesized siRNA analyzed on a 4% E-Gel, which were used for functional testing; 19B) measurements of luciferase activities after transfection of cells with lacZ siRNA; 19C) measurements of  $\beta$ -galactosidase activities after transfection of cells with lacZ siRNA
- [0087] Figures 20A-20B show purification of siRNA generated with Dicer and RNaseIII.
- [0088] Figure 21 shows functional testing of siRNA preparations with FipIn293-luc cells. Relative luciferase activity was measured for siRNA samples.
- [0089] Figures 22A-22B show functional testing of siRNA preparations with GripTite™ 293 MSR cells. 22A) Beta-galactosidase assay: Effect of luc siRNA and lacZ siRNA generated with Dicer and RNaseIII enzyme on  $\beta$ -galactosidase activity. 22B) Luciferase assay: Effect of luc siRNA and lacZ siRNA generated with Dicer and RNaseIII enzyme on luciferase activity.
- [0090] Figures 23A-23B show determination of column capacity and recovery efficiency. A) Recovery of tRNA after binding to the column matrix with a

single 100- $\mu$ l or two 50- $\mu$ l elutions. B) Recovery of a 1-kb dsRNA fragment after binding to the column matrix with a single 100- $\mu$ l or two 50- $\mu$ l elutions.

[0091] Figures 24A-24B show clean-up of long dsRNA and tRNA A) Clean-up of 100-, 500-, and 1000-bp fragments of dsRNA. B) Clean-up of yeast tRNA.

## DETAILED DESCRIPTION OF THE INVENTION

### I. Definitions

[0092] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0093] As used herein, the term "animal" is meant to include any animal, including but not limited to worms (*e.g.*, *C. elegans*), insects (including but not limited to, *Drosophila* spp., *Trichoplusa* spp., and *Spodoptera* spp.), fish, reptiles, amphibians, birds (including, but not limited to chickens, turkeys, ducks, geese and the like), marsupials, mammals and the like. Mammals include without limitation cats, large felines (lions, tigers, etc.), dogs, wolves, mice, rats, rabbits, deer, mules, bears, cows, pigs, horses, oxen, zebras, elephants, primates, and humans.

[0094] As used herein, the term "gene" refers to a nucleic acid comprising an open reading frame encoding a polypeptide (a structural gene), or a sequence that is the reverse complement of a gene product that is a nucleic acid, typically an RNA molecule (including without limitation ribosomal RNA, tRNA, Micro-RNAs and the like), including both exon and (optionally) intron sequences.

[0095] As used herein, the term "regulation of gene expression" refers to the act of controlling the ability of a gene to produce a biologically active protein. Regulation may result in increased expression of a gene, decreased expression of a gene or maintenance of expression of a gene, as described herein.

[0096] As used herein, the term "plurality" refers to more than one.

[0097] As used herein when referring to any numerical value, the term "about" means a value of  $\pm 10\%$  of the stated value. For example, "about 50°C" encompasses a range of temperatures from 45°C to 55°C, inclusive; similarly, "about 100 mM" encompasses a range of concentrations from 90 mM to 110 mM, inclusive.

[0098] A liquid solution that is "substantially free of" a substance comprises less than about 5 to 10% of the substance, preferably less than about 1 to 5%, more preferably less than about 0.1 to 1%, most preferably less than about 0.1%, by volume. A solid that is "substantially free of" a substance comprises less than about 5% of the substance, preferably less than about 1%, more preferably less than about 0.1%, by weight.

[0099] The terms "separate", "isolate" and "purify" have the following meanings herein. A compound of interest is said to have been separated from a mixture of other compounds if the separation process results in the mixture being enriched for the compound of interest or substantially free of at least one of the other compounds. Separation can be partial (*e.g.*, as in fractionation). Purification signifies that the compound of interest is substantially free of other, chemically dissimilar types of compounds; for example, nucleic acids are purified from mixtures comprising proteins, lipids, carbohydrates, etc. Isolation results in a compound that is in pure form, *i.e.*, free or substantially free from all other compounds, whether chemically similar or not. It should be noted that these processes are not mutually exclusive and need not occur in any particular order or association linked. For example, an isolated compound of interest can be prepared by separating the compound from, *e.g.*, 10 other compounds in a mixture; if the compound of interest is separated from compounds of different types, it is said to have been purified (or partially purified). Separation, followed by various degrees of purification, is one way to effect isolation of a compound of interest. However, distinct steps are not always used, as it may be possible to prepare in some instances to isolate an isolated compound from a mixture of compounds in a single step. The term



“preparing” includes but is not limited to separating, isolating, purifying, enriching and fractionating, whether performed as a method *per se*, a step in a method, or in combination with other methods.

[0100] A “weak buffer” is a buffer that has low electrical conductivity and/or low ionic strength. Conductivity is reciprocal of electrical resistivity, which may be measured using a conductivity meter including without limitation commercially meters such as those sold by Hanna Instruments (Bedfordshire, U.K.), ICM (Hillsboro, OR), and Orion meters (MG Scientific, Pleasant Prairie, WI).

[0101] As used herein, “low electrical conductivity” indicates a conductivity of from about 0.1 mS.cm<sup>-1</sup> to about 1,000 mS.cm<sup>-1</sup>; from about 0.1 mS.cm<sup>-1</sup> to about 500 mS.cm<sup>-1</sup>; from about 0.1 mS.cm<sup>-1</sup> to about 250 mS.cm<sup>-1</sup>; from about 0.1 mS.cm<sup>-1</sup> to about 100 mS.cm<sup>-1</sup>; from about 0.1 mS.cm<sup>-1</sup> to about 50 mS.cm<sup>-1</sup>; from about 0.1 mS.cm<sup>-1</sup> to about 10 mS.cm<sup>-1</sup>; from about 0.1 mS.cm<sup>-1</sup> to about 5 mS.cm<sup>-1</sup>; from about 0.1 mS.cm<sup>-1</sup> to about 1 mS.cm<sup>-1</sup>; and from about 0.1 mS.cm<sup>-1</sup> to about 0.5 mS.cm<sup>-1</sup>.

[0102] Ionic strength (  $I$  ) is calculated according to the following formula and rules:

$$I = \frac{1}{2} \sum_i z_i^2 [X_i]$$

wherein  $z_i$  is the charge on the ion  $i$  at a molar concentration  $[X_i]$ . Uncharged species do not contribute to ionic strength. If the solution comprises more than one type of salt or buffering species, the ionic strength contributions of each species must be summed in order to determine  $I$  for the solution.

[0103] As used herein, “low ionic strength” indicates an ionic strength of from about 1 micromho/cm to about 10,000 micromho/cm; from about 1 micromho/cm to about 5,000 micromho/cm; from about 1 micromho/cm to about 1,000 micromho/cm; from about 1 micromho/cm to about 500 micromho/cm; from about 1 micromho/cm to about 100 micromho/cm; from about 1 micromho/cm to about 50 micromho/cm; from about 1 micromho/cm

to about 10 micromho/cm; or from about 1 micromho/cm to about 5 micromho/cm.

[0104] Examples of weak buffers include without limitation TE buffer (10 mM Tris-HCl and 1 mM EDTA), 2xTE buffer (20 mM Tris-HCl and 2 mM EDTA), 3xTE buffer (30 mM Tris-HCl and 3 mM EDTA), TE-plus buffer (from about 30 to about 100 mM Tris-HCl, and from about 1 to about 10 mM EDTA), and from about 2-fold to about 100-fold dilutions thereof. Another example is phosphate-buffered saline (PBS) buffer (*e.g.*, from about 0.7% to about 0.1% NaCl and from about 1 to about 10 mM sodium phosphate), such as D-PBS (Dulbecco's Phosphate-Buffered Saline), and from about 2-fold to about 100-fold dilutions thereof. Such buffers preferably have a pH of from about 6 to about 8, from about 6.5 to about 7.5, or about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, about 6.6, about 6.7, about 6.8, about 6.9, about 7.0, about 7.1, about 7.2, about 7.3, about 7.4, about 7.5, about 7.6, about 7.7, about 7.8, about 7.9, or about 8.0.

## II. Exemplary Embodiments

[0105] The present invention provides methods of separating, isolating and/or purifying Short RNA molecules that are between from about 10 and about 30 nucleotides or base pairs in length. In various embodiments, the RNA molecules are double-stranded (ds). RNAi molecules are one type of Short RNA molecule, and typically comprise from about 12 to about 30 bp, from about 4 to 26, from about 6 to 24 or from 18 to 22 bp. That is, the Short RNA may comprise 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or 31 bp. Preferred Short RNA molecules comprise 19 to 23 bp. Particularly preferred are Short RNA molecules generated by the action of an enzyme called DICER; these Short RNA molecules typically comprise 21, 22 or 23 bp. A Short RNA molecule may comprise both double-stranded (ds) and single-stranded (ss) portions. It should be understood that the invention can be practiced in such a manner so as to yield Short RNA (or other nucleic acids) having a narrow range of sizes (*e.g.*, 21-23 bp), or to yield RNA or other

nucleic acids having a broader range. The range of sizes of RNA or other nucleic acid that can be prepared according to the invention can be described as from about "q" to about "r" bp, wherein "q" is any whole integer  $\geq 10$  and "r" is any whole integer  $> 15$ , with the proviso that  $q > r$ ,  $q < 1,000,000$  and  $r < 1,000,000$ . Thus, the range of nucleic acids that are prepared according to the invention can range, by way of non-limiting example, from about 10 to about 1,000,000 bp, about 100,000 bp, about 10,000 bp, about 1,000 bp or about 100 bp; from about 30 to about 40 bp, about 50 bp, about 100 bp, about 500 bp, about 1,000 bp, about 5,000 bp, about 10,000 bp, about 100,000 bp, or about 1,000,000 bp; from about 100 bp to about 120 bp, about 150 bp, about 200 bp, about 500 bp, about 1,000 bp, about 10,000 bp, about 100,000 bp to about 1,000,000 bp; from about 1,000 to about 1,200 bp, about 1,500 bp, about 2,000 bp, about 5,000 bp, about 50,000 bp, about 100,000 bp, or about 1,000,000 bp; *etc.*

[0106] The present invention also provides methods of regulating expression of one or more genes in a cell or animal, and methods of treating animals, including humans, by using RNAi molecules and other Short RNA molecules, as well as other nucleic acids, prepared using the methods of the present invention.

[0107] In various embodiments of the invention, the Short RNA molecules that are desirably prepared result from the enzymatic digestion of a larger template nucleic acid. For example, a dsRNA template molecule of 230 bp may be treated to produce ten Short RNA molecules, each of which comprises 23 bp. Enzymes suitable for use in digesting RNA molecules into a plurality of fragments, include, but are not limited to ribonucleases such as DICER, ribonuclease A, nuclease S1, ribonuclease T1, and the like. In particular embodiments, DICER is selected as the digestion enzyme to produce a plurality of Short RNA molecules. Ribonucleases particularly useful in practicing the invention include, without limitation, those described in Table 1 (entitled "Non-limiting Examples of Ribonucleases") and members of the RNase III family of ribonucleases (for reviews, see Lamontagne *et al.*, *Curr*

*Issues Mol Biol.* 3:71-78, 2001; Conrad *et al.*, *Int J Biochem Cell Biol.* 34:116-29, 2002; and Srivastava *et al.*, *Indian J Biochem Biophys.* 33:253-60, 1996).

TABLE 1: NON-LIMITING EXAMPLES OF RIBONCLEASES

	ENZYME	ORGANISM	CITATION / SOURCE / ACCESSION NOS.
	DICER	<i>S. pombe</i>	Provost <i>et al.</i> , 2002a (i)
	DICER	<i>Giardia intestinalis</i>	Accession No. gi 27652061 gb AAO17549.1 [27 652061]
	DICER (a.k.a. CARPEL FACTORY, SHORT INTEGUMENTS1; SUSPENSOR1; CARPEL FACTORY; DCL1)	<i>Arabidopsis thaliana</i>	Park <i>et al.</i> , 2002 (ii); Golden <i>et al.</i> , 2002 (iii); Schauer <i>et al.</i> , 2002 (iv); Accession No. (v)
	DICER (a.k.a. K12H4.8; dcr-1)	<i>Caenorhabditis elegans</i>	Ketting <i>et al.</i> , 2001 (vi); Accession Nos. gi 25145329 ref NP_501019.2 [25 145329]; gi 17552834 ref NP_498761.1 [17 552834]; gi 17539846 ref NP_501018.1 [17 539846]; and gi 21431882 sp P34529 DCR1_C AEEL[21431882]
	DICER	<i>Mus musculus</i>	Nicholson <i>et al.</i> , 2002 (vii); Accession Nos. gi 22507359 ref NP_683750.1 [22 507359]; gi 28522452 ref XP_127160.3 [28 522452]; gi 19072784 gb AAL84637.1 AF4 84523_1[19072784]; gi 24418363 sp Q8R418 DICE_M OUSE[24418363]; gi 22830885 dbj BAC15765.1 [22 830885]; and gi 20385913 gb AAM21495.1 AF

			430845_1[20385913]
	DICER	<i>Mus musculus</i> <i>x Mus spretus</i>	Accession No. gi 19072786 gb AAL84638.1 AF4 84524_1[19072786]
	DICER	<i>Rattus</i> <i>norvegicus</i>	Accession Nos. gi 27719453 ref XP_235831.1 [27 719453]; gi 27668581 ref XP_216776.1 [27 668581]
	DICER	<i>Drosophila</i> <i>melanogaster</i>	Bernstein <i>et al.</i> , 2001 (viii); Accession Nos. gi 16215719 dbj BAB69959.1 [16 215719]
	DICER	<i>Homo sapiens</i>	Matsuda <i>et al.</i> (ix); Accession No. gi 29294651 ref NP_803187.1 [29 294651]; gi 29294649 ref NP_085124.2 [ 29294649; gi 24418367 sp Q9UPY3 DICE _HUMAN[24418367]
	DICER	Human – recombinant	Provost <i>et al.</i> , 2002b (x); Meyers, 2003 (xi) Kawasaki <i>et al.</i> , 2003 (xii)
	RNase III	<i>Escherichia</i> <i>coli</i>	Yang <i>et al.</i> , 2002 (xiii)
	RNase III	<i>Mus musculus</i>	Fortin <i>et al.</i> , 2002 (xiv)
	RNase III	<i>Rhodobacter</i> <i>capsulatus</i>	Rauhut <i>et al.</i> , 1996 (xv)

[0108] References and notes for Table 1: (i) Provost *et al.*, *Proc Natl Acad Sci USA* 99:16648-53, 2002; (ii) Park *et al.*, *Curr Biol* 12:1484-95, 2002; (iii) Golden *et al.*, *Plant Physiol.* 130:808-22, 2002; (iv) Schauer *et al.*, *Trends Plant Sci.* 7:487-91, 2002; (v) Accession Nos. for the Entrez Nucleotides database, which is a collection of sequences from several sources, including the GenBank, RefSeq, and PDBGenBank databases, which can be accessed on-line at <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>; (vi) Ketting *et al.*, *Genes Dev.* 15:2654-9, 2001; (vii) Nicholson *et al.*, *Mamm Genome* 13:67-73, 2002; (viii) Bernstein *et al.*, *Nature* 409:363-6, 2001; (ix) Matsuda *et al.*, *Biochim Biophys Acta* 1490:163-9, 2000; (x) Provost *et al.*, *EMBO J.* 21:5864-74, 2002; (xi) Myers *et al.*, *Nat Biotechnol.* 21:324-8, 2003; (xii) Kawasaki *et al.*, *Nucleic Acids Res.* 31:981-7, 2003; (xiii) Yang *et al.*, *Proc Natl Acad Sci USA* 99:9942-9947, 2002; (xiv) Fortin *et al.*, *BMC Genomics* 3:26, 2002; and Rauhut *et al.*, *Nucleic Acids Res.* 24:1246-1251, 1996.

[0109] Affinity columns that may be used in the methods of the invention include, but are not limited to glass fiber RNA purification columns (*e.g.*, such as those in the Micro-to-Midi Total RNA Purification kits, Invitrogen Corp., Carlsbad, CA), Sephadex™ columns, Sepharose™ columns, Superdex™ columns, Superose™ columns (Amersham Biosciences Corp., Piscataway, NJ), ion exchange chromatography (IEX) columns (Amersham; Princeton Chromatography, Cranbury, NJ), and the like.

[0110] Alcohols that may be used in the present invention include, but are not limited to, methanol; ethanol; propanol; isopropanol; butanol; isobutyl alcohol; tertiary butyl alcohol; 1-, 2- and 3-hexanol; and the like. Alcohols that are miscible with water are generally preferred. Combinations of alcohols can also be used. In some applications, including but not limited to those in which it is desirable to keep the volume of the sample low, isopropanol is preferably used in place of ethanol.

[0111] In some embodiments, the alcoholic solution is an azeotrope, a liquid mixture of two or more substances that retains the same composition in the vapor state as in the liquid state when distilled or partially evaporated under a

certain pressure. An azeotrope is thus a mixture that has its own unique boiling point that is different (lower) than those of its components. For example, an azeotrope of ethanol and water comprises about 4% water and about 96% ethanol, depending on the pressure; under ambient conditions, the ethanol:water azeotrope is about 4.4% water and about 95.6% ethanol. As another example, an azeotrope of isopropanol and water comprises about 13% water and about 87% isopropanol, depending on the pressure; under ambient conditions, the isopropanol:water azeotrope is about 12.6% water and about 87.4% ethanol. As is known in the art, the composition of an azeotrope may change depending on what other compounds are present in the solution. For example, a mixture of a buffer with ethanol may form an azeotrope having a composition different from about 4% water and about 96% ethanol. Other parameters (*e.g.*, atmospheric pressure) may also influence the composition of any given azeotrope. One skilled in the art will be able to determine, either empirically or by calculation using known formulae, the composition of a specific azeotrope under any particular set of circumstances, and will also be able to prepare azeotropes directly (*e.g.*, by distillation).

[0112] In some embodiments of the invention, the first fluid mixture comprises ethanol, suitably in the range of about 1 to about 50% ethanol by volume. For example, the first fluid mixture may comprise between from about 5 to about 50%, about 10 to about 40%, or about 20 to about 40% ethanol by volume. In other particular embodiments, the second fluid mixture comprises ethanol, suitably in the range of about 50 to about 100% ethanol. For example, the second fluid mixture may comprise about 50 to about 95%, about 60 to about 90%, or about 60 to about 80% ethanol by volume.

[0113] In various embodiments, the first and/or second fluid mixtures may also comprise a suitable physiologic buffer. Physiologic buffers that may be used in the methods of the present invention include, but are not limited to, those comprising saline, Tris-(hydroxymethyl)aminomethane-HCl (TRIS-HCl), Ethylene-diaminetetraacetic acid (EDTA) disodium salt, Phosphate Buffered Saline (PBS), N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic

acid) (HEPES), 3-(N-Morpholino)propanesulfonic acid (MOPS), 2-bis(2-Hydroxyethylene)amino-2-(hydroxymethyl)-1,3-propanediol (bis-TRIS), potassium phosphate ( $\text{KPO}_4$ ), sodium phosphate ( $\text{NaPO}_4$ ), dibasic sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ), monobasic sodium phosphate ( $\text{NaH}_2\text{PO}_4$ ), monobasic sodium potassium phosphate ( $\text{NaKHPO}_4$ ), magnesium phosphate ( $\text{Mg}_3(\text{PO}_4)_2 \cdot 4\text{H}_2\text{O}$ ), potassium acetate ( $\text{CH}_3\text{COOH}$ ), D(+)- $\alpha$ -sodium glycerophosphate ( $\text{HOCH}_2\text{CH}(\text{OH})\text{CH}_2\text{OPO}_3\text{Na}_2$ ) and other physiologic buffers known to those skilled in the art. These first and second fluid mixtures may also comprise additional reagents including, but not limited to, guanidine isothiocyanate,  $\beta$ -mercaptoethanol and other reducing agents, and the like.

[0114] In various embodiments of the methods of the invention, the first fluid mixture comprises isopropanol, suitably in the range of about 1 to about 50% isopropanol by volume. For example, the first fluid mixture may comprise between from about 5 to about 50%, about 10 to about 40%, or about 20 to about 40% isopropanol by volume. In other embodiments, the second fluid mixture comprises isopropanol, suitably in the range of about 50 to about 100% isopropanol. For example, the second fluid mixture may comprise about 50 to about 95%, about 60 to about 90%, or about 60 to about 80% isopropanol by volume. In various embodiments, the first and/or second fluid mixtures may also comprise a suitable physiologic buffer and/or additional reagents as described above.

[0115] The third fluid mixture used in the preparative methods of the invention is substantially free of alcohol (e.g., ethanol or isopropanol). In various embodiments, the third fluid mixture will not contain any alcohol. In additional embodiments, the third fluid mixture may contain a weak and/or dilute physiologic buffer. Suitable physiologic buffers include those described above.

[0116] In preferred embodiments, the third fluid mixture will be a weak buffer as that term is herein described.

[0117] Varying the ethanol concentrations at each binding step will cause different sized Short RNA molecules to bind or pass through the membranes.



These concentrations can be optimized to obtain Short RNA molecules of a preferred purity and/or concentration.

[0118] In particular embodiments, the methods of the present invention provide a method of isolating one or more RNA molecules, comprising: (a) obtaining one or more RNA molecules; (b) digesting these one or more RNA molecules to produce a plurality of Short RNA molecules; (c) adding water or an aqueous solution, such as a buffer, which may be a weak buffer, comprising between from about 1 to about 50% ethanol by volume to the plurality of Short RNA molecules to produce an RNA fragment solution; (d) loading the RNA fragment solution onto one or more first affinity columns; (e) passing the RNA fragment solution through these one or more first affinity columns, and collecting Short RNA molecules that are less than about 30 nucleotides or base pairs in length with the eluate; (f) adding a buffer solution comprising between from about 50 to about 100% ethanol to the eluate; (g) loading the eluate onto one or more second affinity columns and allowing the solution to pass through the one or more second affinity columns, wherein Short RNA molecules that are between from about 10 and about 30 nucleotides or base pairs in length bind to the one or more second affinity columns; (h) adding a buffer solution comprising between from about 50 to about 100% ethanol to the same one or more second affinity columns and allowing the solution to pass through the columns, wherein Short RNA molecules that are between from about 10 and about 30 nucleotides or base pairs in length remain bound to the one or more affinity columns; (i) adding water or an aqueous solution, such as a buffer, which may be a weak buffer, to the one or more second affinity columns thereby eluting the Short RNA molecules from the one or more second affinity columns; and (j) collecting one or more Short RNA molecules that are between from about 10 and about 30 nucleotides or base pairs in length.

[0119] Suitable digestion enzymes for use in this embodiment of the invention include, but are not limited to DICER, ribonuclease A, nuclease S1, ribonuclease T1, and the like. In particular embodiments, DICER is selected

as the digestion enzyme to produce a plurality of Short RNA molecules, including but not limited to RNAi molecules.

[0120] Affinity columns that may be used in this embodiment of the invention include, but are not limited to glass fiber RNA purification columns (Micro-to-Midi Total RNA Purification kit, Invitrogen Corp., Carlsbad, California), Sephadex™, Sepharose™, Superdex™, Superose™ (Amersham Biosciences Corp., Piscataway, NJ), IEX columns, and the like. In other embodiments of this method of the invention, isopropanol, or another alcohol (*e.g.* methanol, butanol, etc.), or combinations thereof, may be substituted for ethanol.

[0121] Elution of Short RNA molecules and solutions through the affinity columns may occur simply via gravity, may be facilitated through the use of a centrifuge to spin the columns, or by positive or negative (vacuum) pressure. In embodiments using glass fiber filters, centrifugation and/or positive or negative pressure are preferred.

[0122] In certain embodiments of the invention, step (h), which may be described as a "washing" step, may be repeated multiple times (*i.e.* two, five, 10, 20, etc. times) prior to eluting the bound Short RNA molecules that are between from about 10 and about 30 nucleotides or base pairs in length with water or a dilute buffer in step (i). In other embodiments of the invention, the isolation methods do not have to comprise the RNA digestion step (b) as described above and may simply comprise the isolation of Short RNA molecules between from about 10 and about 30 nucleotides or base pairs in length from a plurality of RNAi molecules.

[0123] In suitable embodiments of the invention, the methods are used to prepare Short RNA molecules between from about 10 and about 30 nucleotides or base pairs in length for use as interfering RNA. Suitable nucleic acid molecules are Short RNA molecules, including without limitation RNAi molecules, which can be separated, isolated and/or purified using the methods of the present invention.

### III. Short RNA Molecules and Other Nucleic Acids

[0124] As used herein, the term “nucleic acids” (which is used herein interchangeably and equivalently with the term “nucleic acid molecules”) refers to nucleic acids (including DNA, RNA, and DNA-RNA hybrid molecules) that are isolated from a natural source; that are prepared *in vitro*, using techniques such as PCR amplification or chemical synthesis; that are prepared *in vivo*, *e.g.*, via recombinant DNA technology; or that are prepared or obtained by any appropriate method. Nucleic acids used in accordance with the invention may be of any shape (linear, circular, etc.) or topology (single-stranded, double-stranded, linear, circular, supercoiled, torsional, nicked, etc.). The term “nucleic acids” also includes without limitation nucleic acid derivatives such as peptide nucleic acids (PNAs) and polypeptide-nucleic acid conjugates; nucleic acids having at least one chemically modified sugar residue, backbone, internucleotide linkage, base, nucleotide, nucleoside, or nucleotide analog or derivative; as well as nucleic acids having chemically modified 5' or 3' ends; and nucleic acids having two or more of such modifications. Not all linkages in a nucleic acid need to be identical.

[0125] Nucleic acids can be synthesized *in vitro*, prepared from natural biological sources (*e.g.*, cells, organelles, viruses and the like), or collected as an environmental or other sample. Examples of nucleic acids include without limitation oligonucleotides (including but not limited to antisense oligonucleotides), ribozymes, aptamers, polynucleotides, artificial chromosomes, cloning vectors and constructs, expression vectors and constructs, gene therapy vectors and constructs, PNA (peptide nucleic acid) DNA and RNA.

[0126] RNA includes without limitation rRNA, mRNA, and Short RNA. As used herein, the term “Short RNA” encompasses RNA molecules described in the literature as “tiny RNA” (Storz, *Science* 296:1260-3, 2002; Illangasekare *et al.*, *RNA* 5:1482-1489, 1999); prokaryotic “small RNA” (sRNA) (Wassarman *et al.*, *Trends Microbiol.* 7:37-45, 1999); eukaryotic “noncoding

RNA (ncRNA)”; “micro-RNA (miRNA)”; “small non-mRNA (snmRNA)”; “functional RNA (fRNA)”; “transfer RNA (tRNA)”; “catalytic RNA” [*e.g.*, ribozymes, including self-acylating ribozymes (Illangaskare et al., RNA 5:1482-1489, 1999); “small nucleolar RNAs (snoRNAs)”; “tmRNA” (a.k.a. “10S RNA”, Muto *et al.*, *Trends Biochem Sci.* 23:25-29, 1998; and Gillet *et al.*, *Mol Microbiol.* 42:879-885, 2001); RNAi molecules including without limitation “small interfering RNA (siRNA)”, “endoribonuclease-prepared siRNA (e-siRNA)”, “short hairpin RNA (shRNA)”, and “small temporally regulated RNA (stRNA)”; “diced siRNA (d-siRNA)”, and aptamers, oligonucleotides and other synthetic nucleic acids that comprise at least one uracil base.

### III.A. Oligonucleotides

[0127] As used in the present invention, an oligonucleotide is a synthetic or biologically produced molecule comprising a covalently linked sequence of nucleotides which may be joined by a phosphodiester bond between the 3' position of the pentose of one nucleotide and the 5' position of the pentose of the adjacent nucleotide. As used herein, the term “oligonucleotide” includes natural nucleic acid molecules (*i.e.*, DNA and RNA) as well as non-natural or derivative molecules such as peptide nucleic acids, phosphorothioate-containing nucleic acids, phosphonate-containing nucleic acids and the like. In addition, oligonucleotides of the present invention may contain modified or non-naturally occurring sugar residues (*e.g.*, arabinose) and/or modified base residues. The term oligonucleotide encompasses derivative molecules such as nucleic acid molecules comprising various natural nucleotides, derivative nucleotides, modified nucleotides or combinations thereof. Oligonucleotides of the present invention may also comprise blocking groups which prevent the interaction of the molecule with particular proteins, enzymes or substrates.

[0128] Oligonucleotides include without limitation RNA, DNA and hybrid RNA-DNA molecules having sequences that have minimum lengths of *e* nucleotides, wherein “*e*” is any whole integer from about 2 to about 15, and

maximum lengths of about  $f$  nucleotides, wherein " $f$ " is any whole integer from about 2 to about 200. In general, a minimum of about 6 nucleotides, preferably about 10, and more preferably about 12 to about 15 nucleotides, is desirable to effect specific binding to a complementary nucleic acid strand.

[0129] In general, oligonucleotides may be single-stranded (ss) or double-stranded (ds) DNA or RNA, or conjugates (*e.g.*, RNA molecules having 5' and 3' DNA "clamps") or hybrids (*e.g.*, RNA:DNA paired molecules), or derivatives (chemically modified forms thereof). Single-stranded DNA is often preferred, as DNA is less susceptible to nuclease degradation than RNA. Similarly, chemical modifications that enhance the specificity or stability of an oligonucleotide are preferred in some applications of the invention.

[0130] Certain types of oligonucleotides are of particular utility in the compositions and complexes of the present invention, including but not limited to RNAi molecules, antisense oligonucleotides, ribozymes, and aptamers.

### III.A.1. Antisense Oligonucleotides

[0131] Nucleic acid molecules suitable for use in the present invention include antisense oligonucleotides. In general, antisense oligonucleotides comprise nucleotide sequences sufficient in identity and number to effect specific hybridization with a preselected nucleic acid. Antisense oligonucleotides are generally designed to bind either directly to mRNA transcribed from, or to a selected DNA portion of, a targeted gene, thereby modulating the amount of protein translated from the mRNA or the amount of mRNA transcribed from the gene, respectively. Antisense oligonucleotides may be used as research tools, diagnostic aids, and therapeutic agents.

[0132] Antisense oligonucleotides used in accordance with the present invention typically have sequences that are selected to be sufficiently complementary to the target mRNA sequence so that the antisense oligonucleotide forms a stable hybrid with the mRNA and inhibits the translation of the mRNA sequence, preferably under physiological conditions.

It is preferred but not necessary that the antisense oligonucleotide be 100% complementary to a portion of the target gene sequence. However, the present invention also encompasses the production and use of antisense oligonucleotides with a different level of complementarity to the target gene sequence, *e.g.*, antisense oligonucleotides that are at least about  $g\%$  complementary to the target gene sequence, wherein  $g$  is any whole integer from 50 to 100. In certain embodiments, the antisense oligonucleotide hybridizes to an isolated target mRNA under the following conditions: blots are first incubated in prehybridization solution (5x SSC; 25 mM NaPO<sub>4</sub>, pH 6.5; 1x Denhardt's solution; and 1% SDS) at 42°C for at least 2 hours, and then hybridized with radiolabelled cDNA probes or oligonucleotide probes (1x 10<sup>6</sup> cpm/ml of hybridization solution) in hybridization buffer (5x SSC; 25 mM NaPO<sub>4</sub>, pH 6.5; 1x Denhardt's solution; 250 ug/ml total RNA; 50% deionized formamide; 1% SDS; and 10% dextran sulfate). Hybridization for 18 hours at 30-42°C is followed by washing of the filter in 0.1-6x SSC, 0.1% SDS three times at 25-55°C. The hybridization temperatures and stringency of the wash will be determined by the percentage of the GC content of the oligonucleotides in accord with the guidelines described by Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2nd edition, 1989, Cold Spring Harbor Laboratory Press, Plainview, New York), including but not limited to Table 11.2 therein.

- [0133] Representative teachings regarding the synthesis, design, selection and use of antisense oligonucleotides include without limitation U.S. Patent No. 5,789,573, Antisense Inhibition of ICAM-1, E-Selectin, and CMV IE1/IE2, to Baker *et al.*; U.S. Patent No. 6,197,584, Antisense Modulation of CD40 Expression, to Bennett *et al.*; and Ellington, 1992, *Current Protocols in Molecular Biology*, 2nd Ed., Ausubel *et al.*, eds., Wiley Interscience, New York, Units 2.11 and 2.12.

### III.A.2. Ribozymes

[0134] Nucleic acid molecules suitable for use in the present invention also include ribozymes. In general, ribozymes are RNA molecules having enzymatic activities usually associated with cleavage, splicing or ligation of nucleic acid sequences. The typical substrates for ribozymes are RNA molecules, although ribozymes may catalyze reactions in which DNA molecules (or maybe even proteins) serve as substrates. Two distinct regions can be identified in a ribozyme: the binding region which gives the ribozyme its specificity through hybridization to a specific nucleic acid sequence (and possibly also to specific proteins), and a catalytic region which gives the ribozyme the activity of cleavage, ligation or splicing. Ribozymes which are active intracellularly work in cis, catalyzing only a single turnover, and are usually self-modified during the reaction. However, ribozymes can be engineered to act in trans, in a truly catalytic manner, with a turnover greater than one and without being self-modified. Owing to the catalytic nature of the ribozyme, a single ribozyme molecule cleaves many molecules of target RNA and therefore therapeutic activity is achieved in relatively lower concentrations than those required in an antisense treatment (see published PCT patent application WO 96/23569).

[0135] Representative teachings regarding the synthesis, design, selection and use of ribozymes include without limitation U.S. Patent No. 4,987,071 (RNA ribozyme polymerases, dephosphorylases, restriction endoribonucleases and methods) to Cech *et al.*; and U.S. Patent No. 5,877,021 (B7-1 Targeted Ribozymes) to Stinchcomb *et al.*; the disclosures of all of which are incorporated herein by reference in their entireties.

### 3. Nucleic Acids for RNAi (RNAi Molecules)

[0136] Nucleic acid molecules suitable for use in the present invention also include nucleic acid molecules, particularly oligonucleotides, useful in RNA interference (RNAi). In general, RNAi is one method for analyzing gene

function in a sequence-specific manner. For reviews, see Tuschl, *ChemBiochem.* 2:239-245 (2001), and Cullen, *Nat Immunol.* 3:597-599 (2002). RNA-mediated gene-specific silencing has been described in a variety of model organisms, including nematodes (Parrish *et al.*, *Mol Cell* 6:1077-1087, 2000; Tabara *et al.*, *Cell* 99:123-132, 1999); in plants, *i.e.*, "co-suppression" (Napoli *et al.*, *Plant Cell* 2:279-289, 1990) and post-transcriptional or homologous gene silencing (Hamilton *et al.*, *Science* 286:950-952, 1999; Hamilton *et al.*, *EMBO J* 21:4671-4679, 2002) (PTGS or HGS, respectively) in plants; and in fungi, *i.e.*, "quelling" (Romano *et al.*, *Mol Microbiol* 6:3343-3353, 1992). Examples of suitable interfering RNAs include siRNAs, shRNAs and stRNAs. As one of ordinary skill will readily appreciate, however, other RNA molecules having analogous interfering effects are also suitable for use in accordance with this aspect of the present invention.

### III.A.3.a. Small Interfering RNA (siRNA)

[0137] RNAi is mediated by double stranded RNA (dsRNA) molecules that have sequence-specific homology to their "target" mRNAs (Caplen *et al.*, *Proc Natl Acad Sci USA* 98:9742-9747, 2001). Biochemical studies in *Drosophila* cell-free lysates indicates that the mediators of RNA-dependent gene silencing are 21-25 nucleotide "small interfering" RNA duplexes (siRNAs). Accordingly, siRNA molecules are advantageously used in the compositions, complexes and methods of the present invention. The siRNAs are derived from the processing of dsRNA by an RNase known as DICER (Bernstein *et al.*, *Nature* 409:363-366, 2001). It appears that siRNA duplex products are recruited into a multi-protein siRNA complex termed RISC (RNA Induced Silencing Complex). Without wishing to be bound by any particular theory, it is believed that a RISC is guided to a target mRNA, where the siRNA duplex interacts sequence-specifically to mediate cleavage in a catalytic fashion (Bernstein *et al.*, *Nature* 409:363-366, 2001; Boutla *et al.*, *Curr Biol* 11:1776-1780, 2001).



[0138] RNAi has been used to analyze gene function and to identify essential genes in mammalian cells (Elbashir *et al.*, *Methods* 26:199-213, 2002; Harborth *et al.*, *J Cell Sci* 114:4557-4565, 2001), including by way of non-limiting example neurons (Krichevsky *et al.*, *Proc Natl Acad Sci USA* 99:11926-11929, 2002). RNAi is also being evaluated for therapeutic modalities, such as inhibiting or block the infection, replication and/or growth of viruses, including without limitation poliovirus (Gitlin *et al.*, *Nature* 418:379-380, 2002) and HIV (Capodici *et al.*, *J Immunol* 169:5196-5201, 2002), and reducing expression of oncogenes (*e.g.*, the bcr-abl gene; Scherr *et al.*, *Blood* Sep 26 epub ahead of print, 2002). RNAi has been used to modulate gene expression in mammalian (mouse) and amphibian (*Xenopus*) embryos (respectively, Calegari *et al.*, *Proc Natl Acad Sci USA* 99:14236-14240, 2002; and Zhou, *et al.*, *Nucleic Acids Res* 30:1664-1669, 2002), and in postnatal mice (Lewis *et al.*, *Nat Genet* 32:107-108, 2002), and to reduce transgene expression in adult transgenic mice (McCaffrey *et al.*, *Nature* 418:38-39, 2002). Methods have been described for determining the efficacy and specificity of siRNAs in cell culture and *in vivo* (see, *e.g.*, Bertrand *et al.*, *Biochem Biophys Res Commun* 296:1000-1004, 2002; Lassus *et al.*, *Sci STKE* 2002(147):PL13, 2002; and Leirdal *et al.*, *Biochem Biophys Res Commun* 295:744-748, 2002).

[0139] Molecules that mediate RNAi, including without limitation siRNA, can be produced *in vitro* by chemical synthesis (Hohjoh, *FEBS Lett* 521:195-199, 2002), hydrolysis of dsRNA (Yang *et al.*, *Proc Natl Acad Sci USA* 99:9942-9947, 2002), by *in vitro* transcription with T7 RNA polymerase (Donzeet *et al.*, *Nucleic Acids Res* 30:e46, 2002; Yu *et al.*, *Proc Natl Acad Sci USA* 99:6047-6052, 2002), and by hydrolysis of double-stranded RNA using a nuclease such as *E. coli* RNase III (Yang *et al.*, *Proc Natl Acad Sci USA* 99:9942-9947, 2002).

[0140] References regarding siRNA: Bernstein *et al.*, *Nature* 409:363-366, 2001; Boutla *et al.*, *Curr Biol* 11:1776-1780, 2001; Cullen, *Nat Immunol.* 3:597-599, 2002; Caplen *et al.*, *Proc Natl Acad Sci USA* 98:9742-9747, 2001;

Hamilton *et al.*, *Science* 286:950-952, 1999; Nagase *et al.*, *DNA Res.* 6:63-70, 1999; Napoli *et al.*, *Plant Cell* 2:279-289, 1990; Nicholson *et al.*, *Mamm. Genome* 13:67-73, 2002; Parrish *et al.*, *Mol Cell* 6:1077-1087, 2000; Romano *et al.*, *Mol Microbiol* 6:3343-3353, 1992; Tabara *et al.*, *Cell* 99:123-132, 1999; and Tuschl, *Chembiochem.* 2:239-245, 2001.

### III.A.3.b. Short Hairpin RNAs (shRNAs)

- [0141] Paddison *et al.* (*Genes & Dev.* 16:948-958, 2002) have used small RNA molecules folded into hairpins as a means to effect RNAi. Accordingly, such short hairpin RNA (shRNA) molecules are also advantageously used in the methods, compositions and kits of the invention. The length of the stem and loop of functional shRNAs varies; stem lengths can range anywhere from about 25 to about 30 nt, and loop size can range between 4 to about 25 nt without affecting silencing activity. While not wishing to be bound by any particular theory, it is believed that these shRNAs resemble the dsRNA products of the DICER RNase and, in any event, have the same capacity for inhibiting expression of a specific gene.

### III.A.3.c. Small Temporally Regulated RNAs (stRNAs)

- [0142] Another group of small RNAs suitable for use in the compositions, complexes and methods of the present invention are the small temporally regulated RNAs (stRNAs). In general, stRNAs comprise from about 20 to about 30 nt (Banerjee *et al.*, *Bioessays* 24:119-129, 2002). Unlike siRNAs, stRNAs downregulate expression of a target mRNA after the initiation of translation without degrading the mRNA.

### III.A.3.d. Design and Synthesis of siRNA, shRNA, stRNA, Antisense and Other Oligonucleotides

- [0143] One or more of the following guidelines may be used in designing the sequence of siRNA and other nucleic acids designed to bind to a target

mRNA, *e.g.*, shRNA, stRNA, antisense oligonucleotides, ribozymes, and the like, that are advantageously used in accordance with the present invention.

[0144] In the sequence of the target mRNA, select a region located from about 50 to about 100 nt 3' from the start codon. In this region, search for the following sequences: AA(N<sub>19</sub>)TT or AA(N<sub>21</sub>), where N = any nucleotide. The GC content of the selected sequence should be from about 30% to about 70%, preferably about 50%. In order to maximize the specificity of the RNAi, it may be desirable to use the selected sequence in a search for related sequences in the genome of interest; sequences absent from other genes are preferred. The secondary structure of the target mRNA may be determined or predicted, and it may be preferable to select a region of the mRNA that has little or no secondary structure, but it should be noted that secondary structure seems to have little impact on RNAi. When possible, sequences that bind transcription and/or translation factors should be avoided, as they might competitively inhibit the binding of an siRNA, shRNA or stRNA (as well as other antisense oligonucleotides) to the mRNA. Thus, in general, it is preferred to select regions that do not overlap the start codon, and to also avoid the 5' and 3' untranslated regions (UTRs) of an mRNA transcript.

[0145] Nucleic acids that mediate RNAi may be synthesized in vitro using methods to produce oligonucleotides and other nucleic acids, as is described elsewhere herein, and as described in published international Patent Application No. WO 02/061034; U.S. Provisional Patent Application No. 60/254,510, filed December 8, 2000; U.S. Provisional Patent Application No. 60/326,092, filed September 28, 2001; U.S. Patent Application No. 10/014,128, filed December 7, 2001; and U.S. Provisional Patent Application No. 60/520,946, filed November 17, 2003, entitled "Compositions and Methods for Rapidly Generating Recombinant Nucleic Acid Molecules," attorney docket No. INVIT1290-3; the disclosures of which applications are incorporated by reference herein in their entireties. In addition, dsRNA and other molecules that mediate RNAi are available from commercial vendors, such as Ribopharma AG (Kulmach, Germany), Eurogentec (Seraing, Belgium)

and Sequitur (Natick, MA). Eurogentec offers siRNA that has been labeled with fluorophores (*e.g.*, HEX/TET; 5' Fluorescein, 6-FAM; 3' Fluorescein, 6-FAM; Fluorescein dT internal; 5' TAMRA, Rhodamine; 3' TAMRA, Rhodamine), and these are examples of fluorescent dsRNA that can be used in the invention.

#### III.A.4. Aptamers

[0146] Traditionally, techniques for detecting and purifying target molecules have used polypeptides, such as antibodies, that specifically bind such targets. Nucleic acids have long been known to specifically bind other nucleic acids (*e.g.*, ones having complementary sequences). However, nucleic acids that bind non-nucleic target molecules have been described and are generally referred to as aptamers (see, *e.g.*, Blackwell *et al.*, *Science* 250:1104-1110, 1990; Blackwell *et al.*, *Science* 250:1149-1152, 1990; Tuerk *et al.*, *Science* 249:505-510, 1990; and Joyce, *Gene* 82:83-87, 1989. Accordingly, nucleic acid molecules suitable for use in the present invention also include aptamers.

[0147] As applied to aptamers, the term "binding" specifically excludes the "Watson-Crick"-type binding interactions (*i.e.*, A:T and G:C base-pairing) traditionally associated with the DNA double helix. The term "aptamer" thus refers to a nucleic acid or a nucleic acid derivative that specifically binds to a target molecule, wherein the target molecule is either (i) not a nucleic acid, or (ii) a nucleic acid or structural element thereof that is bound by the aptamer through mechanisms other than duplex- or triplex-type base pairing.

[0148] In general, techniques for identifying aptamers involve incubating a preselected non-nucleic acid target molecule with mixtures (2 to 50 members), pools (50 to 5,000 members) or libraries (50 or more members) of different nucleic acids that are potential aptamers under conditions that allow complexes of target molecules and aptamers to form. By "different nucleic acids" it is meant that the nucleotide sequence of each potential aptamer may be different from that of any other member, that is, the sequences of the potential aptamers are random with respect to each other. Randomness can be

introduced in a variety of manners such as, *e.g.*, mutagenesis, which can be carried out *in vivo* by exposing cells harboring a nucleic acid with mutagenic agents, *in vitro* by chemical treatment of a nucleic acid, or *in vitro* by biochemical replication (*e.g.*, PCR) that is deliberately allowed to proceed under conditions that reduce fidelity of replication process; randomized chemical synthesis, *i.e.*, by synthesizing a plurality of nucleic acids having a preselected sequence that, with regards to at least one position in the sequence, is random. By "random at a position in a preselected sequence" it is meant that a position in a sequence that is normally synthesized as, *e.g.*, as close to 100% A as possible (*e.g.*, 5'-C-T-T-A-G-T-3'), is allowed to be randomly synthesized at that position (C-T-T-N-G-T, wherein N indicates a randomized position. At a randomized position, for example, the synthesizing reaction contains 25% each of A, T, C and G; or  $x\%$  A,  $w\%$  T,  $y\%$  C and  $z\%$  G, wherein  $x + w + y + z = 100$ . The randomization at the position may be complete (*i.e.*,  $x = y = w = z = 25\%$ ) or stoichastic (*i.e.*, at least one of  $x$ ,  $w$ ,  $y$  and  $z$  is not 25%).

[0149] In later stages of the process, the sequences are increasingly less randomized and consensus sequences may appear; in any event, it is preferred to ultimately obtain an aptamer having a unique nucleotide sequence.

[0150] Aptamers and pools of aptamers are prepared, identified, characterized and/or purified by any appropriate technique, including those utilizing *in vitro* synthesis, recombinant DNA techniques, PCR amplification, and the like. After their formation, target:aptamer complexes are then separated from the uncomplexed members of the nucleic acid mixture, and the nucleic acids that can be prepared from the complexes are candidate aptamers (at early stages of the technique, the aptamers generally being a population of a multiplicity of nucleotide sequences having varying degrees of specificity for the target). The resulting aptamer (mixture or pool) is then substituted for the starting aptamer (library or pool) in repeated iterations of this series of steps. When a limited number (*e.g.*, a pool or mixture, preferably a mixture with less than 10 members, most preferably 1) of nucleic acids having satisfactory specificity is

obtained, the aptamer is sequenced and characterized. Pure preparations of a given aptamer are generated by any appropriate technique (e.g., PCR amplification, in vitro chemical synthesis, and the like).

[0151] For example, Tuerk and Gold (*Science* 249:505-510, 1990) describe the use of a procedure termed "systematic evolution of ligands by exponential enrichment" (SELEX). In this method, pools of nucleic acid molecules that are randomized at specific positions are subjected to selection for binding to a nucleic acid-binding protein (see, e.g., PCT International Publication No. WO 91/19813 and U.S. Pat. No. 5,270,163). The oligonucleotides so obtained are sequenced and otherwise characterized. Kinzler *et al.* (*Nucleic Acids Res.* 17:3645-3653, 1989) used a similar technique to identify synthetic double-stranded DNA molecules that are specifically bound by DNA-binding polypeptides. Ellington *et al.* (*Nature* 346:818-822, 1990) describe the production of a large number of random sequence RNA molecules and the selection and identification of those that bind specifically to specific dyes such as Cibacron blue.

[0152] Another technique for identifying nucleic acids that bind non-nucleic target molecules is the oligonucleotide combinatorial technique described by Ecker *et al.* (*Nuc. Acids Res.* 21:1853, 1993) known as "synthetic unrandomization of randomized fragments" (SURF), which is based on repetitive synthesis and screening of increasingly simplified sets of oligonucleotide analogue libraries, pools and mixtures (Tuerk *et al.*, *Science* 249:505, 1990). The starting library consists of oligonucleotide analogues of defined length with one position in each pool containing a known analogue and the remaining positions containing equimolar mixtures of all other analogues. With each round of synthesis and selection, the identity of at least one position of the oligomer is determined until the sequences of optimized nucleic acid ligand aptamers are discovered.

[0153] Once a particular candidate aptamer has been identified through a SURF, SELEX or any other technique, its nucleotide sequence can be determined (as is known in the art), and its three-dimensional molecular

structure can be examined by nuclear magnetic resonance (NMR). These techniques are explained in relation to the determination of the three-dimensional structure of a nucleic acid ligand that binds thrombin in Padmanabhan *et al.*, *J. Biol. Chem.* 24:17651 (1993); Wang *et al.*, *Biochemistry* 32:1899 (1993); and Macaya *et al.*, *Proc. Nat'l. Acad. Sci. USA* 90:3745 (1993). Selected aptamers may be resynthesized using one or more modified bases, sugars or backbone linkages. Aptamers consist essentially of the minimum sequence of nucleic acid needed to confer binding specificity, but may be extended on the 5' end, the 3' end, or both, or may be otherwise derivatized or conjugated.

#### III.A.5. Oligonucleotide Synthesis

[0154] The oligonucleotides used in accordance with the present invention can be conveniently and routinely made through the well-known technique of solid-phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Other methods for such synthesis that are known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives. By way of non-limiting example, see, *e.g.*, U.S. Patent No. 4,517,338 (Multiple reactor system and method for polynucleotide synthesis) to Urdea *et al.*, and 4,458,066 (Process for preparing polynucleotides) to Caruthers *et al.*; Lyer *et al.*, Modified oligonucleotides--synthesis, properties and applications. *Curr Opin Mol Ther.* 1:344-358, 1999; Verma *et al.*, Modified oligonucleotides: synthesis and strategy for users. *Annu Rev Biochem.* 67:99-134, 1998; Pfliegerer *et al.*, Recent progress in oligonucleotide synthesis. *Acta Biochim Pol.* 43:37-44, 1996; Warren *et al.*, Principles and methods for the analysis and purification of synthetic deoxyribonucleotides by high-performance liquid chromatography. *Mol Biotechnol.* 4:179-199, 1995; Sproat, Chemistry and applications of oligonucleotide analogues. *J Biotechnol.* 41:221-238, 1995; De Mesmaeker *et al.*, Backbone modifications in oligonucleotides and peptide

nucleic acid systems. *Curr Opin Struct Biol.* 5:343-355, 1995; Charubala *et al.*, Chemical synthesis of 2',5'-oligoadenylate analogues. *Prog Mol Subcell Biol.* 14:114-138, 1994; Sonveaux, Protecting groups in oligonucleotide synthesis. *Methods Mol Biol.* 26:1-71, 1994; Goodchild, Conjugates of oligonucleotides and modified oligonucleotides: a review of their synthesis and properties. *Bioconjug Chem.* 1:165-187, 1990; Thuong *et al.*, Chemical synthesis of natural and modified oligodeoxynucleotides. *Biochimie* 67:673-684, 1985; Itakura *et al.*, Synthesis and use of synthetic oligonucleotides. *Annu Rev Biochem.* 53:323-356, 1984; Caruthers *et al.*, Deoxyoligonucleotide synthesis via the phosphoramidite method. *Gene Amplif Anal.* 3:1-26, 1983; Ohtsuka *et al.*, Recent developments in the chemical synthesis of polynucleotides. *Nucleic Acids Res.* 10:6553-6560, 1982; and Kossel, Recent advances in polynucleotide synthesis. *Fortschr Chem Org Naturst.* 32:297-508, 1975.

### III.A.6. Micro-RNAs

- [0155] MicroRNAs (miRNAs) are short non-coding RNAs that play a role in the control of gene expression. It has been estimated that as much as 1% of the human genome may encode miRNA (Lim *et al.*, *Science* 299:1540, 2003). RNAi molecules, such as those described herein, are one type of miRNA; others are known in the art (see, for example, Meli *et al.*, *Int Microbiol.* 4:5-11, 2001; Wassarman *et al.*, *Trends Microbiol.* 7:37-45, 1999; and The Small RNA Database at <http://mbcr.bcm.tmc.edu/smallRNA/smallrna.html>), and include without limitation tRNAs, snoRNAs and tmRNAs.

#### III.A.6.a. Small Nucleolar RNAs (snoRNAs)

- [0156] Small nucleolar RNAs (snoRNAs) are stable RNA species localized in the eukaryotic nucleoli of a broad variety of eukaryotes including fungi, protists, plants and animals. (For reviews, see Peculis *et al.*, *Curr. Opin. Cell Biol.* 6:1413-1415, 1996; Gerbi, *Biochem. Cell. Biol.* 73:845-858, 1995; and Maxwell *et al.*, *Annu. Rev. Biochem.*, 35:897-934, 1995; see also The snoRNA



Database at <http://rna.wustl.edu/snoRNAdb/>). SnoRNAs have been demonstrated to define sites of nucleotide modifications in rRNA, specifically 2'-O-ribose methylation and formation of pseudouridines, and a few snoRNAs are required for cleavage of precursor rRNA.

[0157] Generally, snoRNAs fall into two groups: the box C/D family and the box H/ACA family (Balakin *et al.*, *Cell* 86:823-834, 1996; Ganot *et al.*, *Genes Dev.* 11:941-956, 1997). (One exception is the MRP RNA, which is involved in a specific pre-rRNA cleavage reaction, perhaps as a ribozyme; see Maxwell *et al.*, *Annu. Rev. Biochem.* 35:897-934, 1995; Tollervey *et al.*, *Curr. Opin. Cell Biol.* 9:337-342, 1997.) A small number of box C/D snoRNAs are involved in rRNA processing; most, however, are known or predicted to serve as guide RNAs in ribose methylation of rRNA.

[0158] The DNA coding units for the snoRNAs occur in both traditional and novel genetic arrangements. Some are transcribed from independent promoters which serve mono- or polycistronic snoRNA coding units. Others are encoded within introns of protein (or protein-like) genes. Regardless of the diverse genomic organization, snoRNA synthesis appears to involve a number of pathways with some common steps: (1) folding of the precursor to form a box C/D or box H/ACA protein binding motif; (2) binding of one or more proteins to this motif; (3) processing of the precursor to the mature RNA; (4) partial or complete assembly of the snoRNP particle; and (5) transport to the nucleolus (Samarsky *et al.*, *EMBO J.* 17:3747-3757, 1998, and references cited therein).

### III.A.6.b. tmRNA

[0159] As the name implies, tmRNA (earlier called "10S RNA") has properties of tRNA and mRNA combined in a single molecule. Acting both as a tRNA and an mRNA, in a process known as trans-translation, tmRNA adds a short peptide tag to undesirable proteins. Trans-translation plays at least two physiological roles: removing ribosomes stalled upon mRNA, and targeting the resulting truncated proteins for degradation by proteases. For a review of

tmRNA, see Muto *et al.*, *Trends Biochem Sci.* 23:25-29 (1998). Sequences of tmRNA molecules may be found in the tmRNA website, <http://www.indiana.edu/~tmrna/>; see also Williams, *Nucleic Acids Res* 27:165-166, 1999; and Williams *et al.*, *Nucleic Acids Res* 26:163-165, 1998.

### III.A.7. Chemical Modifications of Nucleic Acids

[0160] In certain embodiments, particularly those involving synthetic nucleic acids, oligonucleotides used in accordance with the present invention may comprise one or more chemical modifications. By way of non-limiting example, Braasch *et al.* (*Biochemistry* 42:7967-75, 2003) report that RNAi molecules at least tolerate, and may be enhanced by, phosphorothioate linkages and/or the incorporation of 2'-deoxy-2'-fluorouridine. Chemical modifications include with neither limitation nor exclusivity base modifications, sugar modifications, and backbone modifications. In addition, a variety of molecules, including by way of non-limiting example fluorophores and other detectable moieties, can be conjugated to the oligonucleotides or incorporated therein during synthesis. Other suitable modifications include but are not limited to base modifications, sugar modifications, backbone modifications, and the like.

#### III.A.7.a. Base Modifications

[0161] In certain embodiments, the oligonucleotides used in the present invention can comprise one or more base modifications. For example, the base residues in aptamers may be other than naturally occurring bases (*e.g.*, A, G, C, T, U, and the like). Derivatives of purines and pyrimidines are known in the art; an exemplary but not exhaustive list includes aziridinylcytosine, 4-acetylcytosine, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, inosine (and derivatives thereof), N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 7-methylguanine, 3-methylcytosine, 5-methylcytosine (5MC),

N6-methyladenine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5-methoxyuracil, 2-methylthio-N-6-isopentenyladenine, uracil-5-oxyacetic acid methylester, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid, and 2,6-diaminopurine. In addition to nucleic acids that incorporate one or more of such base derivatives, nucleic acids having nucleotide residues that are devoid of a purine or a pyrimidine base may also be included in oligonucleotides and other nucleic acids.

#### III.A.7.b. Sugar Modifications

[0162] The oligonucleotides used in the present invention can also (or alternatively) comprise one or more sugar modifications. For example, the sugar residues in oligonucleotides and other nucleic acids may be other than conventional ribose and deoxyribose residues. By way of non-limiting example, substitution at the 2'-position of the furanose residue enhances nuclease stability. An exemplary, but not exhaustive list, of modified sugar residues includes 2' substituted sugars such as 2'-O-methyl-, 2'-O-alkyl, 2'-O-allyl, 2'-S-alkyl, 2'-S-allyl, 2'-fluoro-, 2'-halo, or 2'-azido-ribose, carbocyclic sugar analogs, alpha-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside, ethyl riboside or propylriboside.

#### III.A.7.c. Backbone Modifications

[0163] The oligonucleotides used in the present invention can also (or alternatively) comprise one or more backbone modifications. For example, chemically modified backbones of oligonucleotides and other nucleic acids include, by way of non-limiting example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates

including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Chemically modified backbones that do not contain a phosphorus atom have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages, including without limitation morpholino linkages; siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; and amide backbones.

#### IV. DICER Reactions

[0164] As reported by Zhang *et al.* (*EMBO J* 21:5875-5885, 2002) and Provost *et al.* (*EMBO J* 21:5864-5874, 2002), activity of recombinantly-produced human DICER is stimulated by limited proteolysis, and the proteolysed enzyme becomes active at 4°C. Cleavage of dsRNA by purified DICER is ATP independent. Complexes of DICER and dsRNA formed at high KCl concentrations are catalytically inactive, which suggests that ionic interactions are involved in dsRNA cleavage. Zhang *et al.* (2002) report that the maximal activity was found at pH 6.5-6.9, 1-5 mM  $Mg^{++}$ , and 50-100 mM NaCl, although it should be noted increasing the NaCl to 0.2 M inhibited the reactions. Other reaction conditions are described in Tuschl *et al.* (*Genes Dev.* 13:3191-3197, 1999) and Zamore *et al.* (*Cell* 101:25-33, 2000).

[0165] Binding of a dsRNA substrate to the enzyme can be uncoupled from the cleavage step by omitting  $Mg^{++}$  or by performing the reaction at 4°C. Thus, it is possible to set up DICER reaction mixes in which the dsRNA

substrate is bound to the enzyme (e.g., due the absence of  $Mg^{++}$ ), but the reaction does not initiate until the DICER is made active (e.g., by the addition of  $Mg^{++}$ ). Similarly, DICER reactions can be terminated by the addition of a chelating agent (e.g., EDTA) in an amount sufficient to lower the concentration of  $Mg^{++}$  to a level insufficient to support the enzymatic reaction, or by addition of an amount of KCl sufficient to inhibit the reaction.

[0166] Conditions for reactions using RNase III have been described. For example, Li *et al.* (*Nucleic Acids Res* 21:1919-1925, 1993) describe reaction conditions for RNase III purified to homogeneity from an overexpressing bacterial strain. For example, one set of reaction conditions is 37°C, in buffer containing 250 mM potassium glutamate and 10 mM  $MgCl_2$ . The magnesium ion ( $Mg^{++}$ ) can be replaced by  $Mn^{++}$  or  $Co^{++}$ , whereas neither  $Ca^{++}$  nor  $Zn^{++}$  support RNase III activity. Li *et al.* (1993) further report that RNase III does not require a monovalent salt for its activity; however, the *in vitro* reactivity pattern is influenced by the monovalent salt concentration. Franch *et al.* (*J Biol Chem* 274:26572-26578, 1999) describe assays of RNase III activity in 1x TMK-glutamate buffer (20 mM Tris acetate, 10 mM magnesium acetate and 200 mM potassium glutamate), which may be supplemented with 1 mM dithiothreitol and 1 ug tRNA, in a reaction volume of 20 ul.

[0167] One skilled in the art will know how to prepare, characterize and assay other RNases from other biological systems. For example, Bellofatto *et al.* (*J Biol Chem* 258:5467-5476, 1983) describe the purification and characterization of an RNA processing enzyme from *Caulobacter crescentus* that has an absolute requirement for monovalent cations. Methods for assaying ribonuclease activity are known. For example, March *et al.* (*Nucleic Acids Res* 18:3293-3298, 1990) describe experiments in which enzyme activity was monitored by assaying fractions for the ability to correctly process exogenous RNA containing specific RNase III cleavage sites

[0168] Following the completion of a dicing reaction, the reaction mixture is diluted with a suitable amount (which may be none) of water or an aqueous solution, such as a buffer, which may be a weak buffer. A suitable amount encompasses an amount of solution determined to be appropriate for the purposes of carrying on the filtration and isolation methods. Such amounts can be readily determined by one skilled in the art, and are encompassed by the present invention. In one embodiment, water or an aqueous solution, such as a buffer, which may be a weak buffer, is added to the reaction mixture to produce a buffered RNA fragment solution. Suitable solutions and buffers include those buffers described throughout this application. In one such embodiment, this solution comprises about 1 to about 10 M guanidine isothiocyanate, about 10 to about 100 mM Tris-HCl (pH 7.0 to 8.0, preferably 7.5), about 1 to about 50mM EDTA (pH 7.5 to 8.5, preferably 8.0), and about 1 to about 10%  $\beta$ -mercaptoethanol. In certain such embodiments, this solution comprises about 4 M guanidine isothiocyanate, about 50 mM Tris-HCl (pH 7.0 to 8.0, preferably 7.5), about 25 mM EDTA (pH 8.0), and about 1%  $\beta$ -mercaptoethanol. This solution typically also comprises ethanol at a final concentration of between from about 1 and about 50% by volume. For example, this solution may comprise between from about 5 to about 50%, about 10 to about 40%, or about 20 to about 40% ethanol by volume. In a certain such embodiment, this solution comprises about 33% ethanol by volume. In other suitable embodiments, isopropanol may be substituted for ethanol.

[0169] The RNA fragment solution is then loaded on one or more first affinity columns. In a suitable embodiment, these one or more first affinity columns are glass fiber RNA purification columns (Micro-to-Midi Total RNA Purification kit, Invitrogen). The eluate comprising Short (*i.e.*, diced) RNA molecules is then allowed to pass through the column and is collected. This eluate may pass through the column via gravity, the column may be centrifuged to facilitate elution or by vacuum or pressure, and combinations of

such methods. In one embodiment, the first affinity columns are centrifuged for 2 minutes at about 10,000 revolutions per minute in a microcentrifuge.

[0170] The eluate comprising the Short RNA molecules is then diluted with a suitable volume of ethanol such that the final concentration of ethanol is between from about 50 and about 100% by volume. For example, the final ethanol concentration may be about 50 to about 95%, about 60 to about 90%, or about 60 to about 80% ethanol by volume. In a certain such embodiment, the volume of ethanol added to the eluate comprising Short RNA molecules is such that the final concentration of ethanol is about 70% by volume. In other suitable embodiments, isopropanol may be substituted for ethanol.

[0171] This diluted eluate comprising the Short RNA molecules is then loaded onto one or more second affinity columns. In a suitable embodiment, these one or more second affinity columns are glass fiber RNA purification columns (Micro-to-Midi Total RNA Purification kit, Invitrogen). Under these conditions, Micro-RNA molecules that are between from about 10 and about 30 nucleotides or base pairs in length bind to the one or more affinity columns. The eluate comprising Short RNA molecules less than about 10 nucleotides or base pairs in length is then allowed to pass through the second affinity columns. This eluate may pass the second affinity columns via gravity, positive or negative (vacuum) pressure, or the columns may be centrifuged to facilitate elution, or combinations of such methods can be used. In a suitable embodiment, the second affinity columns are centrifuged for about 15 seconds at about 10,000 revolutions per minute in a microcentrifuge.

[0172] The one or more second affinity columns are then washed with a suitable buffer comprising between from about 50 to about 100% ethanol by volume. For example, the final ethanol concentration may be about 50 to about 95%, about 60 to about 90%, or about 70 to about 90% ethanol by volume. Suitable buffers include those buffers described throughout this application. In other suitable embodiments, isopropanol may be substituted for ethanol. In suitable embodiments, this wash buffer comprises between 10 to about 50 mM Tris-HCl (pH 7.5), about 0.01 to about 1 mM EDTA (pH 8.0) and about 60 to

about 90% ethanol. In one such embodiment, this was buffer comprises 5mM Tris-HCl (pH 7.5), 0.1 mM EDTA (pH 8.0) and 80% ethanol. In other suitable embodiments, isoproanol may be substituted for ethanol. The eluate is then allowed to pass through the one or more second affinity columns. This eluate may pass through the one or more second affinity columns via gravity, positive or negative (vacuum) pressure, or the columns may be centrifuged to facilitate elution, or combinations of such methods can be used. In a suitable embodiment, the one or more second affinity columns are centrifuged for about 2 minutes at about 10,000 revolutions per minute in a microcentrifuge. This washing step may be repeated multiple times (*i.e.* two, five, 10, 20, etc. times). The one or more second affinity columns are then dried. The columns may be dried via heat, gravity ("drip-dry"), positive or negative (vacuum) pressure, or the columns may be centrifuged, or combinations of such methods can be used. In a suitable embodiment, the second affinity columns are centrifuged for about 2 minutes at about 10,000 revolutions per minute in a microcentrifuge.

[0173] One or more Short RNA molecules is then collected from the second one or more affinity columns. A suitable volume of water or an aqueous solution, such as a buffer, which may be a weak buffer, is added to the second one or more affinity columns and the eluate comprising the one or more Short RNA molecules is collected. This eluate may pass the second affinity columns via gravity, positive or negative (vacuum) pressure, or the columns may be centrifuged to facilitate elution, or combinations of such methods can be used. In a suitable embodiment, the one or more second affinity columns are centrifuged for about 2 minutes at about 10,000 revolutions per minute in a microcentrifuge.

#### V. Gene Regulation, Genomics, Proteomics and High Throughput Screening

[0174] In certain embodiments, RNAi molecules and other nucleic acids (including without limitation antisense nucleic acids) prepared according the present invention are used in various methods of and compositions for gene



regulation. Decreased expression of a gene results in lesser amounts of the final gene product that is, in some embodiments, a protein; however, as is known in the art, some gene products are RNA molecules (*e.g.*, rRNA, tRNA, and the like).

[0175] In any event, RNAi molecules and other nucleic acids are prepared via the invention can be used to decrease expression of one or more target genes. The expression of known genes and proteins is down-regulated in order to observe the effects on a biological system (cells, organisms, etc.) on the loss of the function of a previously identified gene product. For example, if the target gene is a repressor of the expression of other genes, up-regulation of these genes would result from down-regulation of the target gene. In other embodiments, the nucleic acid sequence of a gene is known, but the function of the gene product is unknown; in this case, down-regulation is used to determine the function *per se* of the target gene. The latter embodiment describes, in general terms, the field of proteomics. In both of these embodiments, target nucleic acid sequences are identified and used to design RNAi molecules or other nucleic acids of the invention, and these nucleic acids are prepared using the methods of the invention.

[0176] Partial or complete down-regulation of target genes are possible using RNAi molecules or other nucleic acids prepared via the methods, compositions and kits of the invention. Any measurable degree of down-regulation can be achieved using the invention. The expression of the target gene can be reduced from about 1% to about 99%, with 100% being complete suppression/inhibition of the target gene, although a reduction of anything less than about 5% of expression may not produce a measurable response. In general, the range of down-regulation is any value in the range of from about 5% to 100%. That is, the degree of down-regulation is about  $n\%$ , wherein  $n$  is any whole integer between 5 and 100. For example, about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, or about 99% of gene expression of the target gene may be suppressed.

[0177] Depending on the assay, quantitation of the amount of gene expression allows one to determine the degree of inhibition (or enhancement) of gene expression in a cell or animal treated with one or more RNAi molecules, compared to a cell or animal not so treated according to the present invention. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition or enhancement in a smaller fraction of cells or animals (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells or animals). Quantitation of gene expression in a cell or animal may show similar amounts of inhibition or enhancement at the level of accumulation of target mRNA or translation of target protein. The efficiency of inhibition or enhancement may be determined by assessing the amount of gene product in the cell or animal using any method known in the art. For example, mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the interfering RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region. Methods by which to quantitate mRNA and polypeptide sequences are well-known in the art can be found in, for example, Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> edition, Cold Spring Harbor Laboratory Press, Plainview, New York (1989), and other similar manuals.

[0178] The methods of the present invention can be used to regulate expression of genes that are endogenous to a cell or animal using RNAi molecules prepared via the methods, compositions and kits of the invention. An endogenous gene is any gene that is heritable as an integral element of the genome of the animal species. Regulation of endogenous genes by methods of the invention can provide a method by which to suppress or enhance a phenotype or biological state of a cell or an animal. Examples of phenotypes or biological states that can be regulated include, but are not limited to, shedding or transmission of a virus, feed efficiency, growth rate, palatability, prolificacy, secondary sex characteristics, carcass yield, carcass fat content, wool quality, wool yield, disease resistance, post-partum survival and fertility. Additional endogenous genes that can also be regulated by the methods of the

invention include, but are not limited to, endogenous genes that are required for cell survival, endogenous genes that are required for cell replication, endogenous genes that are required for viral replication, endogenous genes that encode an immunoglobulin locus, and endogenous genes that encode a cell surface protein. Other examples of endogenous genes include developmental genes (*e. g.*, adhesion molecules, cyclin kinase inhibitors, Writ family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors), tumor suppressor genes (*e.g.*, APC, BRCA1, BRCA2, MADH4, MCC, NF 1, NF2, RB 1, TP53, and WTI) and enzymes (*e.g.*, ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

[0179] Methods by which to transfect cells with RNAi molecules and other nucleic acids are well known in the art and include, but are not limited to, electroporation, particle bombardment, microinjection, and through the use of transfection agents. Such transfection agents include without limitation those listed in Table 2 (entitled "Non-limiting Examples of Transfection Agents"), and can be used alone or in combination with each other.

Table 2: Non-limiting Examples of Transfection Agents

TRANS- FECTION AGENT	DESCRIPTION	PATENTS AND/OR CITATIONS	AVAILABLE FROM
BMOP	N-(2-bromoethyl)-N,N-dimethyl-2,3-bis(9-octadecenyloxy)-propanaminium bromide)		
BMOP:DOPE	1:1 (wt/wt) formulation of N-(2-bromoethyl)-N,N-dimethyl-2,3-bis(9-octadecenyloxy)-propanaminium bromide) (BMOP) and DOPE	Walzem <i>et al.</i> , <i>Poult Sci.</i> 76:882-6, 1997. Transfection of avian LMH-2A hepatoma cells with cationic lipids.	
Cationic polysaccharides	Cationic polysaccharides	Published U.S. patent application 2002/0146826	
CellFECTIN®	1:1.5 (M/M) formulation of N, N1, N11, N111-tetramethyl-N, N1, N11, N111-tetrapalmitylspermine (TM-TPS) and dioleoyl phosphatidylethanolamine (DOPE)	U.S. Patents 5,674,908, 5,834,439 and 6,110,916	Invitrogen (LTI)
CTAB:DOPE	formulation of cetyltrimethyl-ammonium bromide (CTAB) and dioleoylphosphatidylethanolamine (DOPE)		
Cytfectin GSV	2:1 (M/M) formulation of cytofectin GS* and dioleoyl phosphatidyl-ethanolamine (DOPE)		(*Cytofectin GS corresponds to Gilead Sciences' GS 3815)
DC-Cholesterol (DC-Chol)	3,β-N,(N',N'-dimethylaminoethane)-carbamo- yl]cholesterol		
DC-Chol:DOPE	formulation of 3,β-N,(N',N'-dimethylaminoethane)-carbamo- yl]cholesterol (DC-Chol) and dioleoyl phosphatidyl-ethanolamine (DOPE)	Gao <i>et al.</i> , <i>Biochim. Biophys. Res. Comm.</i> 179:280-285, 1991	

DC-6-14	O,O'-Ditetradecanoyl-N-(alpha-trimethylammonioacetyl)diethanolamine chloride	Kikuchi <i>et al.</i> , <i>Hum Gene Ther</i> 10:947-55, 1999. Development of novel cationic liposomes for efficient gene transfer into peritoneal disseminated tumor.	
DCPE	Dicaproylphosphatidylethanolamine		
DDPES	Dipalmitoylphosphatidylethanolamine 5-carboxyspermylamide	Behr <i>et al.</i> , <i>Proc. Natl. Acad. Sci. USA</i> 86:6982-6986, 1989. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA; EPO published patent application 0 394 111	
DDAB	didoceyl methylammonium bromide		
Dextran and dextran derivatives or conjugates	DEAE-Dextran; Dextran sulfate	Mai <i>et al.</i> , <i>J Biol Chem.</i> 277:30208-30218, 2002. Efficiency of protein transduction is cell type-dependent and is enhanced by dextran sulfate.	
Diquaternary ammonium salts	(examples:) N,N'-dioleyl-N,N,N',N'-tetramethyl-1,2-ethanediamine (TmedEce), N,N'-dioleyl-N,N,N',N'-tetramethyl-1,3-propanediamine (PropEce), N,N'-dioleyl-N,N,N',N'-tetramethyl-1,6-hexanediamine (HexEce), and their corresponding N,N'-dicetyl saturated analogues (TmedAce, PropAce and HexAce)	Rosenzweig <i>et al.</i> , <i>Bioconjug Chem</i> 12:258-63, 2001. Diquaternary ammonium compounds as transfection agents; U.S. Patent 5,994,317	Vical
DLRIE	dilauryl oxypropyl-3-dimethylhydroxyethylammonium bromide	Felgner <i>et al.</i> , <i>Ann NY Acad Sci</i> 772:126-39, 1995. Improved cationic lipid formulations for <i>in vivo</i> gene therapy.	Vical

DMAP	4-dimethylaminopyridine		
DMPE	Dimyristoylphosphatidylethanolamine		
DMRIE	N-[1-(2,3-dimyristyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl) ammonium bromide	Konopka <i>et al.</i> , <i>Biochim Biophys Acta</i> 1312:186-96, 1996. Human immunodeficiency virus type-1 (HIV-1) infection increases the sensitivity of macrophages and THP-1 cells to cytotoxicity by cationic liposomes.	
DMRIE-C	1:1 formulation of N-[1-(2,3-dimyristyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl) ammonium bromide (DMRIE) and cholesterol	U.S. Patents 5,459,127 and 5,264,618, to Felgner, <i>et al.</i> (Vical)	Invitrogen (LTI)
DMRIE:DOPE	formulation of 1, 2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide and dioleoyl phosphatidylethanolamine (DOPE)	San <i>et al.</i> , <i>Hum Gene Ther</i> 4:781-8, 1993. Safety and short-term toxicity of a novel cationic lipid formulation for human gene therapy.	
DOEPC	dioleoylethylphosphocholine		
DOHME	N-[1-(2,3-dioleoyloxy)propyl]-N-[1-(2-hydroxyethyl)]-N,N-dimethylammonium iodide		
DOPC	dioleoylphosphatidylcholine		
DOPC:DOPS	1:1 (wt%) formulation of DOPC (dioleoylphosphatidylcholine) and DOPS		Avanti
DOSPA	2,3-dioleoyloxy-N-[2-(sperminecarboxamidoethyl)]-N,N-di-methyl-1-propanaminium trifluoroacetate		

DOSPA:DOPE	Formulation of 2,3-dioleoyloxy-N-[2-(sperminecarboxamidoethyl)-N,N-di-met- hyl-1-propanaminium trifluoroacetate (DOSPA) and dioleoyl phosphatidyl-ethanolamine (DOPE)	Baccaglini <i>et al.</i> , <i>J Gene Med</i> 3:82-90, 2001. Cationic liposome-mediated gene transfer to rat salivary epithelial cells <i>in vitro</i> and <i>in vivo</i> .	
DOSPER	1,3-Di-Oleoyloxy-2-(6-Carboxy-spermyl)-propylamid	Buchberger <i>et al.</i> , <i>Biochemica</i> 2:7-10, 1996. DOSPER liposomal transfection reagent: a reagent with unique transfection properties.	Roche
DOTAP	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl-ammonium methylsulfate		
DOTMA	N-[1-(2,3-dioleoyloxy)propyl]-n,n,n-trimethylammoniumchloride		
DPEPC	Dipalmitoylethylphosphatidyl-choline		
Effectene	(non-liposomal lipid formulation used in conjunction with a special DNA-condensing enhancer and optimized buffer)	Zellmer <i>et al.</i> , <i>Histochem Cell Biol</i> 115:41-7, 2001. Long-term expression of foreign genes in normal human epidermal keratinocytes after transfection with lipid/DNA complexes.	Qiagen
FuGENE 6		Wiesenhofer <i>et al.</i> , <i>J Neurosci Methods</i> 92:145-52, 1999. Improved lipid-mediated gene transfer in C6 glioma cells and primary glial cells using FuGene.	Roche

GAP-DLRIE:DOPE	N-(3-aminopropyl)-N, N-dimethyl-2,3-bis(dodecyloxy)-1-propaniminium bromide/dioleoyl phosphatidylethanolamine	Stephan <i>et al.</i> , <i>Hum Gene Ther</i> 7:1803-12, 1996. A new cationic liposome DNA complex enhances the efficiency of arterial gene transfer <i>in vivo</i> .	
GS 2888 cytofectin		Lewis <i>et al.</i> , <i>Proc Natl Acad Sci USA</i> 93:3176-81, 1996. A serum-resistant cytofectin for cellular delivery of antisense oligodeoxynucleotides and plasmid DNA.	Gilead Sciences
Lipofectin®	1:1 (w/w) formulation of N-(1-2,3-dioleoyloxypropyl)-N,N,N-triethylammonium (DOTMA) and dioleoylphosphatidylethanolamine (DOPE)	U.S. Patents 4,897,355; 5,208,066; and 5,550,289.	Invitrogen (LTI)
LipofectACE™	1:2.5 (w/w) formulation of dimethyl dioctadecylammonium bromide (DDAB) and dioleoyl phosphatidylethanolamine (DOPE)		Invitrogen (LTI)
LipofectAMINE™	3:1 (w/w) formulation of 2,3-dioleoyloxy-N-[2(spermincarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and dioleoyl phosphatidylethanolamine (DOPE)	U.S. Patent 5,334,761; and U.S. Patents 5,459,127 and 5,264,618, to Felgner, <i>et al.</i> (Vical)	Invitrogen (LTI)
LipofectAMINE™ 2000			Invitrogen (LTI)
LipofectAMINE PLUS™	PLUS and LipofectAMINE™	U.S. Patents 5,736,392 and 6,051,429	Invitrogen/LTI
LipoTAXI®			Stratagene



monocationic transfection lipids	(examples:) 1-deoxy-1-[dihexadecyl(methyl)ammonio]-D-xylitol; 1-deoxy-1-[methyl(ditetradecyl)ammonio]-D-arabinitol; 1-deoxy-1-[dihexadecyl(methyl)ammonio]-D-arabinitol; 1-deoxy-1-[methyl(dioctadecyl)ammonio]-D-arabinitol	Banerjee <i>et al.</i> , <i>J Med Chem</i> 44:4176-85, 2001. Design, synthesis, and transfection biology of novel cationic glycolipids for use in liposomal gene delivery.	
O-Chol	3 beta[1-ornithinamide-carbamoyl] cholesterol	Lee <i>et al.</i> , <i>Gene Ther</i> 9:859-66, 2002. Intraperitoneal gene delivery mediated by a novel cationic liposome in a peritoneal disseminated ovarian cancer model.	
OliogfectAMIN E™			Invitrogen (LTI)
Piperazine based amphiphilic cationic lipids	Piperazine based amphiphilic cationic lipids	U.S. Patents 5,861,397 and 6,022,874	Vical
PolyFect	(activated-dendrimer molecules with a defined spherical architecture)		Qiagen
Protamine	Protamine mixture prepared from, <i>e.g.</i> , salmon, salt herring, <i>etc.</i> ; can be supplied as, <i>e.g.</i> , a sulfate or phosphate.	Sorgi <i>et al.</i> , <i>Gene Ther</i> 4:961-8, 1997. Protamine sulfate enhances lipid-mediated gene transfer.	Sigma
SuperFect	(activated-dendrimer molecules with a defined spherical architecture)	Tang <i>et al.</i> , <i>Bioconjugate Chem.</i> 7:703, 1996. <i>In vitro</i> gene delivery by degraded polyamido-amine dendrimers.; published PCT applications WO 93/19768 and WO 95/02397	Qiagen
Tfx™	N,N,N',N'-tetramethyl-N,N'-bis(2-hydroxyethyl)-2,3-di(oleoyloxy)-1,4-butanediammonium iodide] and DOPE		Promega

TransFast™	N,N [bis (2-hydroxyethyl)-N-methyl-N-[2,3-di(tetradecanoyloxy) propyl] ammonium iodide and DOPE		Promega
TransfectAce			Invitrogen (LTI)
TRANSFECTA M™	5-carboxylspermylglycine dioctadecylamide (DOGS)	Behr <i>et al.</i> , <i>Proc. Natl. Acad. Sci. USA</i> 86:6982-6986, 1989; EPO Publication 0 394 111	Promega
TransMessenger	(lipid-based formulation that is used in conjunction with a specific RNA-condensing enhancer and an optimized buffer; particularly useful for mRNA transfection)		Qiagen
Vectamidine	3-tetradecylamino-N-tert-butyl-N'-tetradecylpropionamidine (a.k.a. diC14-amidine)	Ouahabi <i>et al.</i> , <i>FEBS Lett</i> 414:187-92, 1997. The role of endosome destabilizing activity in the gene transfer process mediated by cationic lipids.	
X-tremeGENE Q2			Roche

[0180] High throughput screening (HTS) typically uses automated assays to search through large numbers of compounds for a desired activity. Typically HTS assays are used to find new drugs by screening for chemicals that act on a particular enzyme or molecule. For example, if a chemical inactivates an enzyme it might prove to be effective in preventing a process in a cell which causes a disease. High throughput methods enable researchers to try out thousands of different chemicals against each target very quickly using robotic handling systems and automated analysis of results.

[0181] As used herein, "high throughput screening" or "HTS" refers to the rapid in vitro screening of large numbers of compounds (libraries); generally tens to hundreds of thousands of compounds, using robotic screening assays.

Ultra high-throughput Screening (uHTS) generally refers to the high-throughput screening accelerated to greater than 100,000 tests per day.

[0182] To achieve high-throughput screening, it is best to house samples on a multicontainer carrier or platform. A multicontainer carrier facilitates measuring reactions of a plurality of candidate compounds simultaneously. Multi-well microplates may be used as the carrier. Such multi-well microplates, and methods for their use in numerous assays, are both known in the art and commercially available. In HTS embodiments, multi-well plates are temporarily or permanently mated to a multi-well filter block comprising the same number of affinity columns of the invention as the number of wells in the mated multi-well plate. The affinity columns in the filter block are aligned with the wells in the multi-well plate, so that a fluid that is passed through a specified affinity column in the filter block winds up in the corresponding specified well. The wells may contain a target gene expression system, a control reporter system or a "blank" control sample that lacks either reporter system. The expression systems may comprise cellular extracts that can effect *in vitro* transcription and, optionally, translation. Alternatively, the expression systems can be cellular systems, *e.g.*, a cell line expressing a target gene of interest.

[0183] Various types of agents can be screened using the HTS embodiments of the invention. Using RNAi molecules that down-regulate expression of a target gene as a non-limiting example, such molecules are prepared using a filter block of the invention and then contacted with an expression system comprising the target gene. RNAi molecules having high specific activities are identified as those that cause the greatest reduction of expression of the target gene. These or other down-regulating molecules are used to observe the effect of down-regulating the target gene in a series of wells, each of which comprises the same target gene expression system and one or more test compounds unique to the well. In this arrangement, one can screen the test compounds for ones that enhance the down-regulation of the target gene or which compensate for the effects of the target gene down-regulation.

[0184] Screening assays may include controls for purposes of calibration and confirmation of proper manipulation of the components of the assay. Blank wells that contain all of the reactants but no member, of the chemical library are usually included. As another example, a known inhibitor (or activator) of an enzyme for which modulators are sought, can be incubated with one sample of the assay, and the resulting decrease (or increase) in the enzyme activity determined according to the methods herein. It will be appreciated that modulators can also be combined with the enzyme activators or inhibitors to find modulators which inhibit the enzyme activation or repression that is otherwise caused by the presence of the known the enzyme modulator.

## VII. Kits

[0185] In other embodiments, the invention provides a kit comprising at least one affinity column, buffer, alcoholic solution, enzyme or any other composition useful for carrying out the invention. The enzyme may be a ribonuclease, such as DICER or RNase III, or a polymerase, such as an RNA polymerase, including without limitation RNA T7 and SP6 RNA polymerases. Kits according to the invention may further comprise one or more transfection agents, such as those listed in Table 2; one or more nucleic acids, such as a pair of primers, a dsRNA substrate or a vector for transcribing double-stranded RNA; an RNA polymerase; one or more co-factors for an enzyme, such as a nucleotide triphosphate (*e.g.*, ATP, GTP, CTP, TTP or UTP); one or more stop solutions, such as a solution comprising a chelating agent (*e.g.*, EDTA or EGTA), which terminates a reaction catalyzed by an enzyme; a nuclease inhibitor, such as an RNase inhibitor; and one or more set of instructions. The set of instructions can comprise instructions for optimizing ribonuclease reactions and/or instructions for preparing RNAi molecules such as siRNA and e-siRNA molecules.

[0186] A suitable buffer for storage of a substrate dsRNA is 10 mM Tris pH 8.0, 20 mM NaCl, and 1 mM EDTA. Human recombinant DICER (hDicer) or other RNases can be stored in 50 mM Tris pH 8.0, 500 mM NaCl, 20%

Glycerol, 0.1% Triton X-100, and 0.1 mM EDTA, and is stable at 4°C for at least about four months.

[0187] Liquid components of kits are stored in containers, which are typically resealable. A preferred container is an Eppendorf tube, particularly a 1.5 ml Eppendorf tube. A variety of caps may be used with the liquid container. Generally preferred are tubes with screw caps having an ethylene propylene O-ring for a positive leak-proof seal. A preferred cap uniformly compresses the O-ring on the beveled seat of the tube edge. Preferably, the containers and caps may be autoclaved and used over a wide range of temperatures (*e.g.*, +120°C to -200°C) including use with liquid nitrogen. Other containers can be used.

[0188] In one embodiment, a kit of the invention is called a "Dicer RNAi Transfection Kit" and comprises 3 separate packages or "modules". (1) The BLOCK-iT™ Dicer Enzyme Module contains 300 ul of Dicer enzyme at 1 U/ul, 10x Dicer reaction buffer (*e.g.*, 500 mM Tris-HCl, pH 8.5, 1.5 mM NaCl and 30 mM MgCl<sub>2</sub>), stop solution (*e.g.*, 0.5 M EDTA, pH 8.0), RNase-free water and optionally, Dicer Dilution Buffer (*e.g.*, 50 mM Tris-HCl, pH 8, 500 mM NaCl and 20% glycerol) and is stored at -20°C. (2) The BLOCK-iT™ siRNA Purification Module contains an RNA Binding Buffer, RNase-free water, a 5x solution of an RNA Wash Buffer, 50x RNA annealing buffer (*e.g.*, 500 mM Tris-HCl, pH 8.0, 1 M DEPC-treated NaCl, and 50 mM DEPC-treated EDTA), one or more RNA spin cartridges or columns, one or more eluate and flow-through recovery tubes, and one or more siRNA collection tubes, and is stored at ambient temperature. (3) Lipofectamine™ 2000 and/or one or more other transfection agents are optionally also included in this embodiment and are stored at 4°C.

[0189] In another embodiment, a kit of the invention is called a "Dicer RNAi Transcription Kit" and comprises 3 separate packages or "modules". (1) The BLOCK-iT™ RNAi Primer Module contains one or more primers for T7 RNA polymerase (*e.g.*, T7amp1, 5'-GATGACTCGTAATACGACTCACTA-3', SEQ ID NO.:1), RNase-free water and a control template for T7

transcription (e.g., plasmid pcDNA1.2™/V5-GW/lacZ DNA). (2) The BLOCK-iT™ RNAi Transcription Module contains 10x Transcription Buffer (e.g., 400 mM Tris-HCl, pH 8.0, 100 mM DTT, 20 mM Spermidine, and 100 mM MgCl<sub>2</sub>), 75 mM dNTPs, T7 enzyme mix (e.g., 4 parts T7 RNA Polymerase at 50 U/ul, 1 part RNaseOUT at 40 U/ul, and 1 part yeast inorganic pyrophosphatase at 0.6 U/ul), DNase I at 1 U/ul, BLOCK-iT T7 Enzyme Mix, and RNase-free water, and is stored at -20°C. (3) The BLOCK-iT™ Long RNAi Purification Module contains an RNA Binding Buffer, RNase-free water, a 5x solution of an RNA Wash Buffer, 50x RNA annealing buffer, one or more RNA spin cartridges or columns, one or more eluate and flow-through recovery tubes, and one or more RNA collection tubes, and is stored at ambient temperature.

[0190] In a related embodiment, a kit of the invention is called a “Dicer RNAi TOPO® Transcription Kit”. In this embodiment, the TOPO® transcription system (Invitrogen) is used for high-yield RNA synthesis. The Dicer RNAi TOPO® Transcription Kit comprises three modules, which are as described above for the Dicer RNAi Transcription Kit, with the exception that the first module further comprises a T7 TOPO® Linker, 10x PCR buffer, PCR forward and reverse primers (e.g., lacZ-fwd2, 5'- ACCAGAAGCGGTGCCGGAAA-3', SEQ ID NO:2, and lacZ-rev2, 5'-CCACAGCGGATGGTTCGGAT-3', SEQ ID NO:3), 40 mM dNTPs and, optionally, a thermostable polymerase suitable for PCR, e.g., *Taq* polymerase. The T7 TOPO® Linker is a double-stranded oligonucleotide covalently bound to topoisomerase. A single copy of the T7 linker will join to either end of *Taq*-generated PCR products in a reaction that takes about 15 min, preferably from about 1 min to less than about 15 min, thus forming a template for secondary PCR and subsequent transcription. The sense and antisense RNA strands are transcribed by T7 RNA polymerase in separate reactions, purified, and annealed to each other. The resulting long dsRNA can be used as a template for an RNase, such as DICER, to generate short siRNA molecules, which can then be purified using the other components of the kit or otherwise.

[0191] When stored as indicated above, the kits and their components are stable for about from 1 month to about 18 months, from about 3 months to about 12 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months or about 11 months.

[0192] The components of the above kit embodiments can also be packaged in a single kit.

[0193] Kit embodiments of the invention can further comprise nucleic acids (primers, vectors, etc.) and enzymes (ligase, Clonase™, topoisomerase, etc.) useful for cloning the dsRNA substrate and/or siRNA products.

[0194] It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

## EXAMPLES

### EXAMPLE 1

#### dsRNA SUBSTRATE PREPARATION

[0195] The plasmids pcDNA1.2/V5/GW-LacZ and pcDNA5-FRT-luc were used as reporter plasmids for beta-galactosidase and luciferase, respectively, in co-transfection studies.

[0196] The pcDNA5-FRT-luc plasmid comprises a CMV promoter that drives expression of a luciferase gene that terminates with a BGH polyA sequence; it also contains a FRT recombination site. In brief, pcDNA5/FRT (Invitrogen)

was digested with EcoRV and XhoI, and the 5048 bp vector fragment was gel purified. The luciferase gene came from pcDNA6T7EMC-luc (Invitrogen), digested with MscI and XhoI. This 1931 bp luciferase fragment was gel purified and ligated to the 5048 bp vector fragment to create pcDNA5/FRT/luc. The correct clone was verified by examining the products of restriction digests.

[0197] The LacZ expression control plasmid pcDNA1.2<sup>TM</sup>/V5-GW/lacZ was made using Multi-site Gateway. The multi-site assembly format was B4-B1-B2-B3. Briefly, pENTR5'-CMV, pENTR-LacZ and pENTR/V5TKpolyA were mixed with the DEST R4R3 plasmid using LR Plus Clonase. The three plasmids in the Multi-site reaction were all created by standard Gateway recombination reactions: (1) the CMV promoter was amplified from pcDNA3.1 using primers flanked with attB4 and attB1 sequences and recombined with pDonr 5'(P4-P1R) to form pENTR5'-CMV; (2) the LacZ gene was amplified from pcDNA3.1-LacZ using attB1 and attB2 flanking primers and recombined with pDonr 221 to create pENTR-LacZ; and (3) the V5-TKpolyA element was amplified from pcDNA3.2 using attB2 and attB3 primers and recombined with pDonr3'(P2-P3R). All the ENTR clones were verified by sequence as well as restriction digest. Additionally, the cloning junctions and portions of the genes in the final vector were verified by sequence analysis prior to the RNAi assays.

[0198] The respective forward and reverse primers used for the amplification of the beta-galactosidase transcription template for dsRNA synthesis are:

lacZ-fwd2 5'- ACCAGAAGCGGTGCCGGA-3' (SEQ ID NO:2),  
and

lacZ-rev2 5'-CCACAGCGGATGGTTCGGAT-3' (SEQ ID NO:3).

[0199] The respective forward and reverse primers used for the amplification of the luciferase transcription template for dsRNA synthesis are:

LucFor2 5'-TGAACATTTTCGCAGCCTACC-3' (SEQ ID NO:4) and  
LucRev2 5'-GGGGCCACCTGATATCCTTT-3' (SEQ ID NO:5).



[0200] Long dsRNA molecules, generated for use as substrates for DICER reactions, were generated using T7-mediated transcription of pcDNA1.2/V5/GW-LacZ and pcDNA5-FRT-luc and the above-described primers.

## EXAMPLE 2

### DICER REACTIONS

[0201] The conditions used were essentially those described by Myers *et al.* (*Nat Biotechnol.* 21:324-8, 2003). Briefly, His-tagged human recombinant DICER (hDicer) was prepared using an expression construct, pFastBac-HisT7 Dicer Baculovirus, essentially as described in Myers *et al.* (2003). The hDicer was incubated in a 20 ul reaction mix containing 1 ug of dsRNA substrate (prepared as in Example 1), 30 mM Hepes pH 8.0, 250 mM NaCl, and 2.5 mM MgCl<sub>2</sub>. It should be noted that 50 mM Tris pH 8.5 can be used instead of 30 mM HEPES, and that a suitable 10X reaction buffer is 500 mM Tris pH 8.5, 1.5 mM NaCl, and 30 mM MgCl<sub>2</sub>. The reactions were incubated at 37°C for either 6 hours or 14-16 hours, and stopped with the addition of 0.4 ul of 0.5 M EDTA pH 8.0 (final concentration, 1 mM EDTA). The dsRNA concentration was quantified by absorbance at 260 nm. Reaction products were examined by separation by gel electrophoresis and staining (*e.g.*, PAGE in a 20% TBE gel stained with ethidium brodime).

## EXAMPLE 3

### 1-COLUMN PREPARATION OF SHORT, DICED RNA

[0202] The 1-column modality of the invention is illustrated in Panel A of Figure 1. One (1) ug of Dicer-treated lacZ siRNA was prepared according to

the single column method and eluted with various EtOH concentrations containing elution buffer to determine optimal ethanol concentration. Since residual long dsRNA and other intermediates of products caused non-specific response of non-specific shutdown translation and initiation of apoptosis, fractions from 5% ethanol elution (lane 3 in Panel B of Figure 1) to 30% ethanol elution (lane 8 in Panel B of Figure 1) were tested for siRNA functional activity, to define the optimal condition for elution. GripTite™ 293 cells were transfected with a mixture of beta-gal reporter and luciferase plasmids with 1.5 ul of each purified samples. A non-purified fraction (lane 2 in Panel B of Figure 1), and chemically synthesized lacZ and GFP siRNA were used as controls.

[0203] At an EtOH concentration of 5 %, template which was 1 kb dsRNA of lacZ gene was still eluted with partially digested and in elution buffer. However, as increased of EtOH concentration, the 1 kb template and other partially processed products were retained by the column, whereas 19-22 bp siRNA molecules were eluted from the column. Elution buffers containing greater than 35% of EtOH showed a decrease siRNA eluted form the column. Elution buffer contain guanidine isothiocyanate, EDTA and 25% EtOH, and lane 7 and 8 (elution buffer containing each of 25% and 30% ethanol) showed almost full recovery of siRNA (panel B of Figure 1) and some portion of 19-22 bp of siRNA start to remain on the column as the ethanol concentration was increased from 35% to 50%.

[0204] As shown in Panel A of Figure 2, luciferase activity was measured to determine the non-specific reduction from fractions containing the products of a crude lacZ-dicing reaction. The results show a significant reduction of luciferase activity, demonstrating a non-specific reduction in luciferase expression. Elution fractions of 5%, 10% and 15 % ethanol, which are indicated as lacZfrac5, lacZfrac10 and lacZfrac15, showed some non-specific reduction. Such non-specific effects of long ds RNA are thought to be mediated by the dsRNA deendent protein kinase (PKR), which phosphorylates and inactivates the translation factor eIF2alpha, leading to a

generalized suppression of protein synthesis and cell death via both non-apoptotic and apoptotic pathways. The activation of PKR by dsRNA has been shown to be length-dependent and dsRNAs of less than 30 nucleotides are unable to activate pKR and full activation required ~80 nucleotide. However, elution fraction of 20%, 25% and 30% ethanol did not show the non-specific effects as showed by the similar luciferase activities of "reporters only" (no siRNA) and luciferase-unrelated GFP siRNA. The fractions of 25% and 30% ethanol containing elutions showed not only full recovery of purified siRNA, but also proper functionality, including no non-specific reductions in expression. Panel B of Figure 2 shows siRNA effects on lacZ expression of fractions that showed only from about 10% to about 20% beta-gal activity, as compared with cells transfected with reporter only or with unrelated GFP siRNA.

## EXAMPLE 4

### 2-COLUMN PREPARATION OF SHORT, DICED RNA

[0205] The 2-column modality of the invention is illustrated in Panel A of Figure 3. To produce a first binding solution, the following first fluid mixture was added to the dicing reaction: 1 volume of binding buffer (4M guanidine isothiocyanate; 50 mM Tris-HCl, pH 7.5; 25 mM EDTA, pH 8.0; and 1%  $\beta$ -mercaptoethanol) and 1 volume of 100% ethanol (~33% final ethanol concentration). The resulting first binding solution was mixed and loaded onto a first affinity column (a glass fiber RNA purification column from the Micro-to-Midi Total RNA Purification kit, Invitrogen, Carlsbad, CA) and spun for about 15 seconds in a microcentrifuge. The flow-through (FT), which contains short RNA, was collected for further steps. This first column, in which relatively long substrate RNA and/or partially diced RNA molecules are retained, was discarded.

- [0206] To produce a second binding solution, the following second fluid mixture was added to the flow-through: 4x volumes (relative to the volume of the original dicing reaction) of 100% ethanol. The final ethanol concentration was ~70% ethanol. The solution was mixed and loaded onto a second glass fiber RNA purification column and spun 15s in a microcentrifuge, and the flow-through was discarded. As small volume columns were used in this experiment, the second binding solution was loaded and centrifuged 500 ul at a time. This second column, within which short RNA molecules are retained, was used in subsequent steps, whereas the flow-through from the second column was discarded.
- [0207] The second affinity column was washed at this point with 500 µl of wash buffer (5mM Tris-HCl, pH 7.5; 0.1mM EDTA, pH 8.0; 80% ethanol). The wash buffer was loaded onto the column, which was then spun in a microfuge for 1 min, and the flow-through was discarded. The washing was repeated, and the second affinity column was spun as above for 1 min to dry the membrane.
- [0208] The second affinity column was placed in a collection tube. An appropriate volume (30-50 ul) of the third fluid mixture (water, certified RNase-free) was loaded onto the column, which was then incubated at ambient temperature for 1 min. The second column was then spun for 1-2 min to produce an elute comprising diced RNA molecules.
- [0209] The RNA molecules were brought to a final concentration of 10 mM Tris-HCl (pH 8.0), 20 mM NaCl, and 1 mM EDTA (pH 8.0) by adding 1.2 ul of a 50X stock buffer solution to 60 ul of pooled eluate. The concentration of the siRNAs was quantified by absorbance at 260 nm, and the adjusted eluate was then stored at -80°C.
- [0210] The purification method was also performed and validated using isopropanol instead of ethanol. In the first step, the same volume of 100% isopropanol replaced the ethanol. However, after the first column, half as much isopropanol was added to the flow-through as ethanol (a volume equal to twice the original reaction size).

[0211] Representative results are shown in Panel B of Figure 3. The figure shows a 20% TBE gel comprising long dsRNA transcripts (dsRNA), which are a dsRNA substrate for DICER, Dicing reactions (not purified), partially purified Dicing reactions (long and short RNAs retained), or Micro-to-Midi purified diced siRNAs (purified d-siRNAs) targeted against luciferase (luc) and GFP. A synthetic siRNA, generated by synthesis rather than by enzymatic diegestion, is in the rightmost lane.

## EXAMPLE 5

### ACTIVITY OF PURIFIED siRNA

[0212] HEK 293 cells stably expressing firefly luciferase (GL2 variant) were cultured in DMEM containing 4 mM L-glutamine, 10% FBS, and 100 ug/ml hygromycin B. GripTite™ 293 cells (Invitrogen, Carlsbad, CA), which have an enhanced ability to attach to surfaces in culture (see U.S. Patents 5,683,903; 5,863,798; and 5,919,636), were used. The cells were cultured in DMEM containing 4 mM L-glutamine, 10% FBS, and 600ug/ml geneticin.

[0213] Cell lines transfected with siRNAs were used in 24-well plates at 30%-50% confluence corresponding on the day of plating to  $0.6$  to  $1 \times 10^5$  cells/well in 0.5 ml. The d-siRNA or synthetic siRNAs were transfected using 1ml of Lipofectamine 2000 per well. In cotransfection experiments, 100 ng of each reporter plasmid was transfected with respective d-siRNA or synthetic siRNAs into 90% confluent GripTite™ 293 cells plated at  $2 \times 10^5$  cells/well. For each well, 2 ul of Lipofectamine 2000 was used per well, and medium was changed after 3 hr to reduce toxicity associated with plasmid transfection.

[0214] The partial purified DICER products were prepared as described above for the purification of diced RNA molecules, except that the concentration of ethanol in the first binding solution was 70% and only one affinity column was used. As a result, longer substrate dsRNA molecules and partially diced

RNA molecules, as well as completely diced RNAs, all bound simultaneously to the column while protein and other reaction components flowed through. The column was washed, and the RNA molecules eluted therefrom, as described above.

[0215] The cells were transfected with different concentrations of double-stranded RNA, partially purified DICER reaction products, purified DICER reaction products, or chemically synthesized RNAi molecules. The target genes in different experiments were those encoding GL2 luciferase (luc) or GFP.

[0216] Transfections were performed using the amounts of the RNAs shown in Table 3 per well of a 24-well poly-D-lysine coated plate. Cells were plated the day before transfection and were approximately 30% confluent at the time of transfection. Lipofectamine™ 2000 (Invitrogen) was used at a concentration of 1 ul per well.

[0217] Twenty-four hours after transfection, the cells were lysed and assayed using Luciferase Assay Reagent (Promega, Madison, WI) essentially according to the manufacturer's instructions. In brief, one to two days after transfection, medium was aspirated from each well of the 24-well plates and replaced with 500 ml cold luciferase lysis buffer (25mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0, 10% v/v glycerol, 0.1% v/v Triton X-100). Plates were then frozen at -80°C for at least 1hr. Samples were thawed for 30 min at RT and 50 ul of each was transferred to black 96-well plates. Luminescence was measured on a MicroLumat Plus luminometer using Winglow v.1.24 software (EG&G Berthold). Either 50 ul of Luciferase Assay Reagent (Promega) or 100 ul Accelerator II (Tropix) were injected per well and readings were taken for 5 sec after a 2 sec delay. Mean activities and standard errors were calculated for duplicate wells. The results are shown in Table 3.

Table 3: RNAi ACTIVITY

TREATMENT	LUCIFERASE ACTIVITY (RLU)	STANDARD ERROR OF MEAN
Untransfected	86,644	3550
mock transfected	85,969	394
luc syn siRNA		
20 ng	16,983	296
50 ng	14,629	30
100 ng	13,187	99
GFP syn siRNA		
20 ng	94,957	313
50 ng	96,673	1163
100 ng	99,581	2147
luc dsRNA*		
20ng	24,618	553
50ng	20,854	346
100ng	18,817	87
GFP dsRNA*		
20ng	24,056	180
50ng	23,167	495
100ng	20,748	752
luc syn siRNA – partially purified		
20 ng	9,899	527
50 ng	8,768	242
100 ng	7,538	433
GFP siRNA – partially purified		
20 ng	20,325	141
50 ng	18,460	98
100 ng	17,706	26

Luc purified diced siRNA		
20 ng	13,488	307
50 ng	12,354	541
100 ng	9,127	357
GFP purified diced siRNA		
20 ng	103,028	1953
50 ng	99,502	2775
100 ng	107,341	3962

\* dsRNA = double-stranded substrate RNA (not "diced")

[0218] Down regulation of luciferase activity by luciferase-targeting RNAi molecules, but not by GFP-targeting RNAs was observed for the synthetic siRNA molecules ("syn siRNA" in Table 3) and purified diced siRNA molecules; thus, the purified diced siRNA molecules and syn siRNA molecules are specific for the targeted gene (luc). Both luciferase and GFP dsRNA substrates and unpurified diced siRNA from intermediate-sized DICER products strongly reduced luciferase activity, indicating a non-specific response by the cells to the larger nucleic acid molecules. Thus, the RNAi molecules prepared according to the invention are specific for their target gene, and non-specific effects are reduced or eliminated.

## EXAMPLE 6

### FRACTIONATION OF dsDNA USING DIFFERENT CONCENTRATIONS OF ETHANOL

[0219] The ethanol gradient modality of the invention is illustrated in Panel A of Figure 4. A 10 bp DNA ladder was used to examine the affinity of dsDNA fragments of various sizes to the glass filters with increasing concentrations of ethanol in the binding buffer. Passing the DNA ladder over a series of Micro-to-Midi columns with 10% stepwise increases in ethanol concentration (and concomitant decreases in the concentration of the other binding buffer



components) allowed for the separation of fragments according to size (Panel B of Figure 4). For example, a significant portion of the 20 bp fragment could be captured along with some contaminating 30 bp fragment to the exclusion of the 10 bp and 40 bp fragments; see Panel B of Figure 3, lane 40 (40% ethanol).

## EXAMPLE 7

### TRANSFECTION OF CELLS BY MICRO-RNA MOLECULES

[0220] The non-limiting examples of transfection agents described in Table 2 can be used to deliver RNAi molecules and other Short RNA molecules. For example, Lipofectamine™ 2000 has been used to transfect siRNA into mammalian cells (Gitlin *et al.*, *Nature* 418:379-380, 2002; Yu *et al.*, *Proc Natl Acad Sci USA* 99:6047-6052, 2002), and Oligofectamine™ has been used to transfect siRNA into HeLa cells (Elbashir *et al.*, *Nature* 411:494-498, 2001; Harborth *et al.*, *J Cell Sci* 114:4557-4565, 2001).

[0221] In general, the following guidelines should be used when using these transfection agents to introduce siRNA into cells. First, the cells should be transfected when they are about 30 to about 50% confluent. Second, antibiotics should not be added during the transfection as this may cause cell death. Third, for optimal results, the transfection agent should be diluted in Opti-MEM® I Reduced Media (Invitrogen) prior to being combined with siRNA.

## EXAMPLE 8

### RNAi TARGETING AN ENDOGENOUS GENE

[0222] The d-siRNA was prepared as in the preceding Examples. The following forward and reverse primers used for the amplification of the lamin A/C transcription template for dsRNA synthesis:

laminAC-fwd, 5'AGGAGAAGGAGGACCTGCAG-3' (SEQ ID NO.:6); and

laminAC-rev, 5'AGAAGCTCCTGGTACTCGTC-3' (SEQ ID NO.:7).

[0223] The PCR template, IMAGE clone 4863480, was a pOTB7-derived plasmid that contains the laminA/C gene. The source of the plasmid was the IMAGE (Integrated Molecular Analysis of Genomes and their Expression) Consortium. The amplification product was about 1kbp in size.

[0224] The human lung carcinoma cell line, A549, was cultured in F-12K media containing 2 mM L-glutamine and 10% FBS and transfected with siRNAs in 24-well plates at 30%-50% confluence corresponding on the day of plating to  $2 \times 10^4$  cells/well in 0.5 ml. The d-siRNA or synthetic siRNAs were transfected using 1 ml of Lipofectamine™ 2000 per well.

[0225] Forty-eight (48) hrs post-transfection, protein extracts were prepared from A549 cells that had not been transfected or transfected with lipid only (mock), synthetic siRNA (siRNA, ~200 ng), or Diced siRNA (d-siRNA). In brief, cell pellets containing lamin A/C were resuspended in protein sample loading buffer and denatured at 95°C for 5 min before separation on a NuPAGE Novex 4-12% Tris-Bis Gel. To facilitate the simultaneous detection of lamin A/C and actin in a single sample, the unstained BenchMark ladder was excised from the blot and stained with MemCode Reagent (Pierce Chemical Co., Rockford, IL) to facilitate cutting the blot between the 50 and 60 kDa molecular weight standards.

[0226] The blot was cut in two for staining with Anti-laminA/C or anti-actin antibody. Western Blot analysis was performed using the Chemiluminescent

Western Breeze Immunodetection Kit (Invitrogen, Carlsbad, CA) essentially according to the manufacturer's instructions. The lamin A/C protein was detected using the lamin A/C Monoclonal Antibody clone 14 (BD Biosciences) at 1:1000. Actin was detected using the beta-actin monoclonal antibody clone AC-15 (Abcam) at 1:5000.

[0227] The results are shown in Figure 4. The synthetic siRNA molecules ("laminA/C siRNA") and diced siRNA molecules ("d-siRNA") caused a marked decrease in the amount of lamin A/C detected on the gel. Cells treated with the lacZ siRNA molecules showed some reduction in lamin A/C; this effect is slight and appears to be specific for the lacZ siRNA molecules as other siRNA molecules do not have this effect. In any event, the lacZ siRNA molecules are "leaky" to the extent where enough lamin A/C remains that no phenotypic change is seen. In contrast, the lamin A/C siRNA molecules cause a more severe down-regulation of the gene.

## EXAMPLE 9

### MULTIWELL FORMAT TRANSFECTION

[0228] The compounds, compositions and methods described herein can be used to transfect cells in a multiwell format, *e.g.*, a 24-, 48-, 96-, or 384-well plate. The following procedures describes the transfection of siRNA into cells using Lipofectamine™ 2000 or Oligofectamine™, and can be adapted to use with any other nucleic acids or transfection agents or combinations thereof.

[0229] In any procedure, one should have the following materials prepared beforehand: siRNA of interest (20 pmol/ul); prewarmed Opti-MEM® I Reduced Media (Invitrogen); and 24-well tissue culture plates and other tissue culture supplies. The cells to be transfected should be about 30 to about 50% confluent, and cell populations are preferably determined before transfection to comprise at least about 90% viable cells.

[0230] The following procedures are used to transfect mammalian cells in a 24-well format. To transfect cells in other tissue culture formats (e.g., 48-, 96- or 384-well plates), optimal conditions for those formats might vary from those given herein for the 24-well format.

#### 6.1. Lipofectamine™ 2000

[0231] For transfecting HEK293, BHK, CHO-1, or A549 cells, see Table 4 for suggested transfection conditions. Typically, in RNAi studies using these conditions, a decrease of >50%, preferably >70%, more preferably >80% decrease, most preferably >95% in the expression of a stably integrated reporter gene or an endogenous gene is observed by about 24 to about 48 hours after transfection.

Table 4: siRNA Transfection Conditions for exemplary Cell Lines

<i>CELL LINE</i>	CELL DENSITY (CELLS/WELL)	AMOUNT OF LIPOFECTAMINE™ 2000	AMOUNT OF siRNA
HEK 293	$1 \times 10^5$	1 $\mu$ l	20 pmol
BHK	$1.5 \times 10^4$	1 $\mu$ l	20 pmol
CHO-K1	$4 \times 10^4$	1 $\mu$ l	20 pmol
A549	$1.5 \times 10^4$	1 $\mu$ l	20 pmol

[0232] 1. One day before transfection, plate cells in 0.5 ml of growth medium without antibiotics so that they will be about 30 to about 50% confluent at the time of transfection.

[0233] 2. For each transfection sample, prepare siRNA:Lipofectamine™ 2000 complexes as follows:

[0234] (a) Dilute the appropriate amount of siRNA in 50  $\mu$ l of Opti-MEM® Reduced Serum Medium without serum (or other medium without serum). Mix gently.

[0235] (b) Mix Lipofectamine™ 2000 gently before use, then dilute the appropriate amount in 50 ul of Opti-MEM® Medium (or other medium without serum). Mix gently and incubate for 5 minutes at room temperature. Note: Combine the diluted Lipofectamine™ 2000 with the diluted siRNA within 30 minutes. Longer incubation times may decrease activity. If D-MEM is used as a diluent for the Lipofectamine™ 2000, mix with the diluted siRNA within 5 minutes.

[0236] (c) After the 5 minute incubation, combine the diluted siRNA with the diluted Lipofectamine™ 2000 (total volume is 100 ul). Mix gently and incubate for 20 minutes at room temperature.

[0237] 3. Add the 100 ul of the siRNA/Lipofectamine™ 2000 mixture to each well. Mix gently by, for example, rocking the plate back and forth.

[0238] 4. Incubate the cells at 37°C in a CO<sub>2</sub> incubator for about 24 to about 72 hours until they are ready to be assayed for gene expression. It is generally not necessary to remove the complexes or change the medium; however, growth medium may be replaced after about 4 to about 6 hours without loss of transfection activity.

## 6.2. Oligofectamine™

[0239] Typically, in RNAi studies of HeLa cells using the following conditions, a decrease of >50%, preferably >70%, more preferably >80% decrease, most preferably >95% in the expression of a stably integrated reporter gene or an endogenous gene is observed by about 24 to about 48 hours after transfection.

[0240] 1. One day before transfection, plate cells in 0.5 ml of growth medium without antibiotics so that they will be about 50% confluent at the time of transfection.

[0241] 2. For each transfection sample, prepare siRNA:Oligofectamine™ complexes as follows:

[0242] (a) Dilute 60 pmol of siRNA in 50 ul of Opti-MEM® Reduced Serum Medium without serum (or other medium without serum). Mix gently.

- [0243] (b) Mix Oligofectamine™ gently before use, then dilute 3 ul in 12 ul of Opti-MEM® Medium (or other medium without serum). Mix gently and incubate for 5 minutes at room temperature.
- [0244] (c) After the 5 minute incubation, combine the diluted siRNA with the diluted Oligofectamine™ (total volume is 68 ul). Mix gently and incubate for 20 minutes at room temperature.
- [0245] 3. Add the 68 ul of the siRNA:Oligofectamine™ mixture to each well. Mix gently by, for example, rocking the plate back and forth.
- [0246] 4. Incubate the cells at 37°C in a CO2 incubator for about 24 to about 72 hours until they are ready to be assayed for gene expression. It is generally not necessary to remove the complexes or change the medium; however, growth medium may be replaced after about 4 to about 6 hours without loss of transfection activity.

### Example 10

- [0247] Exemplary product literature is provided below that describes the generation, purification, and transfection of gene-specific d-siRNA for use in RNA interference analysis, TOPO-mediated generation of templates and production of double-stranded RNA for use in RNA interference analysis. All catalog numbers provided below correspond to Invitrogen Corporation products, Carlsbad, CA, unless otherwise noted.

#### **D-SIRNA GENERATION AND TRANSFECTION PROCEDURE**

##### Produce dsRNA

- [0248] Follow the guidelines to generate dsRNA. If you are using the BLOCK-iT™ Complete Dicer RNAi Kit, refer to the BLOCK-iT™ RNAi TOPO® Transcription Kit manual for instructions to generate dsRNA.

Perform the dicing reaction

- [0249] 1. Set up the following dicing reaction:

10X Dicer Buffer	30 $\mu$ l
RNase-Free Water	up to 210 $\mu$ l
Purified dsRNA (60 $\mu$ g)	1-150 $\mu$ l
BLOCK-iT™ Dicer Enzyme (1U/ $\mu$ l)	60 $\mu$ l
Total volume	300 $\mu$ l

- [0250] 2. Mix reaction gently and incubate for 14-18 hours at 37°C.

- [0251] 3. Add 6  $\mu$ l of 50X Dicer Stop Solution.

- [0252] 4. Check integrity of the d-siRNA, if desired. Proceed to purify d-siRNA.

Purify d-siRNA

- [0253] 1. To each 300  $\mu$ l dicing reaction, add 300  $\mu$ l of RNA Binding Buffer containing 1% (v/v)  $\beta$ -mercaptoethanol followed by 300  $\mu$ l of isopropanol. Mix well by pipetting up and down 5 times.

- [0254] 2. Apply half the sample (~450  $\mu$ l) to the RNA Spin Cartridge, and centrifuge at 14,000 x g for 15 seconds at room temperature. Save the flow-through.

- [0255] 3. Transfer the RNA Spin Cartridge to an siRNA Collection Tube and repeat Step 2, using the other half of the dicing reaction sample (~450  $\mu$ l). Save the flow-through.

- [0256] 4. Transfer the flow-through from Step 2 to the siRNA Collection Tube containing the flow-through from Step 3. Add 600  $\mu$ l of isopropanol and mix well by pipetting up and down 5 times.

- [0257] 5. Apply one-third of the sample (~500  $\mu$ l) to a new RNA Spin Cartridge. Centrifuge at 14,000 x g for 15 seconds at room temperature. Discard the flow-through.

- [0258] 6. Repeat Step 5 twice, applying one-third of the remaining sample (~500  $\mu$ l) to the RNA Spin Cartridge each time.
- [0259] 7. Add 500  $\mu$ l of 1X RNA Wash Buffer to the RNA Spin Cartridge, and centrifuge at 14,000 x g for 15 seconds at room temperature. Discard the flow-through.
- [0260] 8. Repeat Step 7.
- [0261] 9. Centrifuge the RNA Spin Cartridge at 14,000 x g for 1 minute at room temperature.
- [0262] 10. Remove the RNA Spin Cartridge from the Wash Tube and place it in an RNA Recovery Tube.
- [0263] 11. Add 30  $\mu$ l of RNase-Free Water to the RNA Spin Cartridge. Let stand at room temperature for 1 minute, then centrifuge the RNA Spin Cartridge at 14,000 x g for 2 minutes at room temperature to elute the d-siRNA.
- [0264] 12. Add 30  $\mu$ l of RNase-Free Water to the RNA Spin Cartridge and repeat Step 11, eluting the d-siRNA into the same RNA Recovery Tube.
- [0265] 13. Add 1.2  $\mu$ l of 50X RNA Annealing Buffer to the eluted d-siRNA.
- [0266] 14. Quantitate the yield of d-siRNA by spectrophotometry. Aliquot and store the d-siRNA at -80°C.

#### Transfect d-siRNA

- [0267] Follow the procedure below to transfect cells using Lipofectamine™ 2000. Refer to later table for the appropriate reagent amounts and volumes to add for different tissue culture formats.
- [0268] 1. One day before transfection, plate cells in growth medium without antibiotics such that they will be 30-50% confluent at the time of transfection.
- [0269] 2. For each transfection sample, prepare d-siRNA:Lipofectamine™ 2000 complexes as follows:



- (a) Dilute d-siRNA in the appropriate amount of Opti-MEM® I Reduced Serum Medium without serum. Mix gently.
  - (b) Mix Lipofectamine™ 2000 gently before use, then dilute the appropriate amount in Opti-MEM® I. Mix gently and incubate for 5 minutes at room temperature.
  - (c) After the 5 minute incubation, combine the diluted d-siRNA with the diluted Lipofectamine™ 2000. Mix gently and incubate for 20 minutes at room temperature.
- [0270] 3. Add the d-siRNA:Lipofectamine™ 2000 complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.
- [0271] 4. Incubate the cells at 37°C in a CO2 incubator until they are ready to assay for gene knockdown.

#### Control Reaction

- [0272] If you have purchased the BLOCK-iT™ Complete Dicer RNAi Kit, we recommend using the control template and control PCR primers included with the kit to produce dsRNA (see the BLOCK-iT™ RNAi TOPO® Transcription Kit manual for details). Once you have produced dsRNA, use this dsRNA as a control in your dicing, purification, and transfection experiments.

#### Kit Contents and Storage

#### Types of Kits

- [0273] The BLOCK-iT™ Complete Dicer RNAi Kit is also supplied with the BLOCK-iT™ RNAi TOPO® Transcription Kit and the BLOCK-iT™ RNAi TOPO® Transcription Kit manual.

Product	Catalog No.
BLOCK-iT™ Dicer RNAi Transfection Kit	K3600-01

BLOCK-iT™ Complete Dicer RNAi Kit	K3650-01
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#### Kit Components

**[0274]** The BLOCK-iT™ Dicer RNAi Kits include the following components. For a detailed description of the contents of each component, see later description. For a detailed description of the contents of the BLOCK-iT™ RNAi TOPO® Transcription Kit, see the BLOCK-iT™ RNAi TOPO® Transcription Kit manual.

Component	Catalog no.	
	K3600-01	K3650-01
BLOCK-iT™ Dicer Enzyme Kit	√	√
BLOCK-iT™ RNAi Purification Kit	√	√
Lipofectamine™ 2000 Reagent	√	√
BLOCK-iT™ RNAi TOPO® Transcription Kit		√

#### Shipping/Storage

**[0275]** The BLOCK-iT™ Dicer RNAi Kits are shipped as described below. Upon receipt, store each item as detailed below. For more detailed information about the reagents supplied with the BLOCK-iT™ RNAi TOPO® Transcription Kit, refer to the BLOCK-iT™ RNAi TOPO® Transcription Kit manual.

Box	Component	Shipping	Storage
1	BLOCK-iT™ Dicer Enzyme Kit	Dry ice	-20°C
2	BLOCK-iT™ RNAi Purification Kit	Room temperature	Room temperature
3	Lipofectamine™ 2000 Reagent	Wet ice	+4°C (do not freeze)

4-6	BLOCK-iT™ RNAi TOPO® Transcription Kit	BLOCK-iT™ TOPO® Linker Kit and BLOCK-iT™ RNAi Transcription Kit: Dry ice BLOCK-iT™ RNAi Purification Kit: Room temperature	BLOCK-iT™ TOPO® Linker Kit and BLOCK-iT™ RNAi Transcription Kit: -20°C BLOCK-iT™ RNAi Purification Kit: Room temperature
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#### BLOCK-iT™ Dicer Enzyme Kit

[0276] The following reagents are included with the BLOCK-iT™ Dicer Enzyme Kit (Box 1). Store the reagents at -20°C.

Reagent	Composition	Amount
BLOCK-iT™ Dicer Enzyme	1 U/μl in a buffer	300 μl
10X Dicer Buffer		150 μl
50X Dicer Stop Buffer	0.5 mM EDTA, pH 8.0	30 μl
RNase-Free Water	--	1.5 ml

[0277] One unit of BLOCK-iT™ Dicer enzyme cleaves 1 μg of double-stranded RNA (dsRNA) in 16 hours at 37°C.

#### BLOCK-iT™ RNAi Purification Kit

[0278] The following reagents are included with the BLOCK-iT™ RNAi Purification Kit (Box 2). Store reagents at room temperature. Use caution when handling the RNA Binding Buffer.

[0279] Note: Catalog no. K3650-01 includes two boxes of BLOCK-iT™ RNAi Purification reagents. One box is supplied with the BLOCK-iT™ RNAi TOPO® Transcription Kit for purification of sense and antisense single-stranded RNA (ssRNA). The second box is supplied for purification of diced siRNA (d-siRNA).

Reagent	Composition	Amount
RNA Binding Buffer		1.8 ml
5X RNA Wash Buffer		2.5 ml
RNase-Free Water	--	800 µl
RNA Spin Cartridges	--	10
RNA Recovery Tubes	--	10
siRNA Collection Tubes*	--	5
50X RNA Annealing Buffer	500 mM Tris-HCl, pH 8.0 1 M NaCl 50 mM EDTA, pH 8.0	50 µl

\*siRNA Collection Tubes are used for purification of d-siRNA only, and are not required for the purification of the ssRNA.

[0280] The RNA Binding Buffer supplied in the BLOCK-iT™ RNAi Purification Kit contains guanidine isothiocyanate. This chemical is harmful if it comes in contact with the skin or is inhaled or swallowed. Always wear a laboratory coat, disposable gloves, and goggles when handling solutions containing this chemical.

[0281] Do not add bleach or acidic solutions directly to solutions containing guanidine isothiocyanate or sample preparation waste. Guanidine isothiocyanate forms reactive compounds and toxic gases when mixed with bleach or acids.

#### Lipofectamine™ 2000 Reagent

[0282] Each BLOCK-iT™ Dicer RNAi Kit includes Lipofectamine™ 2000 Reagent (Box 3) for high efficiency transfection of d-siRNA into mammalian cells. Lipofectamine™ 2000 Reagent is supplied as follows:

Size: 0.75 ml

Concentration: 1 mg/ml

Storage: +4°C; do not freeze

### BLOCK-iT™ RNAi TOPO® Transcription Kit

- [0283] The BLOCK-iT™ Complete Dicer RNAi Kit (Catalog no. K3650-01) includes the BLOCK-iT™ RNAi TOPO® Transcription Kit to facilitate production of double-stranded RNA (dsRNA) from your gene of interest. Refer to the BLOCK-iT™ RNAi TOPO® Transcription Kit manual for a detailed description of the reagents provided with the kit and instructions to produce dsRNA.

#### Accessory Products

- [0284] The products listed in this section may be used with the BLOCK-iT™ Dicer RNAi Kits.

#### Accessory Products

- [0285] Some of the reagents supplied in the BLOCK-iT™ Dicer RNAi Kits as well as other products suitable for use with the kit are available separately from Invitrogen.

Item	Amount	Catalog no.
BLOCK-iT™ RNAi TOPO® Transcription Kit	5 genes	K3500-01
Lipofectamine™ 2000 Reagent	0.75 ml	11668-027
	1.5 ml	11668-019
Opti-MEM® I Reduced Serum Medium	100 ml	31985-062
	500 ml	31985-070
Phosphate-Buffered Saline (PBS), pH 7.4	500 ml	10010-023
4% E-Gel® Starter Pak	9 gels and Base	G5000-04
20% Novex® TBE Gel	1 box	EC63152BOX
10 bp DNA Ladder	50 µg	10821-015
β-Gal Assay Kit	100 reactions	K1455-01

#### Overview

[0286] The BLOCK-iT™ Dicer RNAi Transfection Kit and the BLOCK-iT™ Complete Dicer RNAi Kit facilitate generation of purified diced siRNA duplexes (d-siRNA) that are suitable for use in RNAi analysis of a target gene in mammalian cells. Both kits contain the BLOCK-iT™ Dicer Enzyme for dicing dsRNA, reagents to purify the d-siRNA, and an optimized transfection reagent for highly efficient delivery of d-siRNA to mammalian cells.

[0287] The BLOCK-iT™ Complete Dicer RNAi Kit also includes the BLOCK-iT™ RNAi TOPO® Transcription Kit to facilitate high-yield generation of purified dsRNA. For more information, refer to the BLOCK-iT™ RNAi TOPO® Transcription Kit manual. This manual is supplied with the BLOCK-iT™ Complete Dicer RNAi Kit.

#### Advantages of the BLOCK-iT™ Dicer RNAi Transfection Kit

**[0288]** Using the BLOCK-iT™ Dicer RNAi Transfection Kit and the BLOCK-iT™ Complete Dicer RNAi Kit to generate d-siRNA for RNAi analysis in mammalian provides the following advantages:

Provides a cost-effective means to enzymatically generate a pool of d-siRNA that cover a larger portion of the target gene (e.g. 500 bp to 1 kb) without the need for expensive chemical synthesis of siRNA.

Provides the BLOCK-iT™ Dicer Enzyme and an optimized protocol to facilitate generation of the highest yields of d-siRNA from a dsRNA substrate.

Includes BLOCK-iT™ RNAi Purification reagents for efficient purification of d-siRNA. Purified d-siRNA can be quantitated, enabling highly reproducible RNAi analysis.

Includes the Lipofectamine™ 2000 Reagent for the highest efficiency transfection in a wide variety of mammalian cell lines.

#### Purpose of this Manual

**[0289]** This manual provides the following information:

A description of the components in the BLOCK-iT™ Dicer RNAi Transfection Kit and an overview of the pathway by which d-siRNA facilitates gene knockdown in mammalian cells.

Guidelines to produce dsRNA corresponding to the target gene. For detailed instructions to produce dsRNA, refer to the BLOCK-iT™ RNAi TOPO® Transcription Kit manual.

Guidelines and instructions to use the BLOCK-iT™ Dicer Enzyme to cleave dsRNA to generate a complex pool of d-siRNA.

Instructions to purify d-siRNA.

Guidelines and instructions to transfect purified d-siRNA into mammalian cells using Lipofectamine™ 2000 Reagent for RNAi studies.

**[0290]** The BLOCK-iT™ Dicer RNAi Transfection Kit and the BLOCK-iT™ Complete Dicer RNAi Kit are designed to help generate d-siRNA for use in

RNAi analysis in mammalian cell lines. Although the kits have been designed to help generate d-siRNA representing a particular target sequence in the simplest, most direct fashion, use of the resulting d-siRNA for RNAi analysis assumes that users are familiar with the principles of gene silencing and transfection in mammalian systems. We highly recommend that users possess a working knowledge of the RNAi pathway and lipid-mediated transfection.

- [0291] For more information about the RNAi pathway in mammalian cells, refer to published reviews (Elbashir, S. M., *et al.*, Methods 26:199-213 (2002); McManus, M.T. and Sharp, P.A., Nature Rev. Genet. 3:737-747 (2002)).

#### BLOCK-iT™ Dicer RNAi Kit

##### Components of the BLOCK-iT™ Dicer RNAi Kit

- [0292] The BLOCK-iT™ Dicer RNAi Transfection Kit and the BLOCK-iT™ Complete Dicer RNAi Kit facilitate generation and delivery of purified d-siRNA duplexes into mammalian cells for RNAi analysis. The kits contain three major components:

The BLOCK-iT™ Dicer Enzyme and optimized reagents for production of high yields of d-siRNA from a dsRNA substrate. For more information about how the BLOCK-iT™ Dicer Enzyme works, below.

The BLOCK-iT™ RNAi Purification reagents for silica-based column purification of d-siRNA, and an RNA Annealing Buffer to stabilize d-siRNA duplexes for long-term storage.

Lipofectamine™ 2000 Reagent for high-efficiency transfection of d-siRNA into a wide range of mammalian cell types and cell lines for RNAi analysis.

- [0293] If you are using the BLOCK-iT™ Complete Dicer RNAi Kit, note that the kit also includes a control expression plasmid containing the lacZ gene and PCR primers that may be used to generate control lacZ dsRNA. The control lacZ dsRNA may be used in a dicing and purification reaction to generate purified lacZ d-siRNA. Co-transfecting the purified lacZ d-siRNA and the



control expression plasmid into mammalian cells provide a means to assess the RNAi response in your cell line by assaying for knockdown of  $\beta$ -galactosidase. In addition, the lacZ d-siRNA can be used as a negative control for non-specific off-target effects in your RNAi studies.

[0294] If you are using the BLOCK-iT™ Complete Dicer RNAi Kit, note that the kit includes 2 boxes of BLOCK-iT™ RNAi Purification reagents. One box is intended for purification of dsRNA, while the second box is intended for purification of d-siRNA. The protocols to purify dsRNA and d-siRNA differ significantly from one another. When purifying d-siRNA, be sure to use the purification procedure provided in this manual. To purify dsRNA, use the purification procedure provided in the BLOCK-iT™ RNAi TOPO® Transcription Kit manual.

#### Generating d-siRNA Using the Kit

[0295] Using the reagents supplied in the kit, you will perform the following steps to generate pure d-siRNA that is ready for transfection into the mammalian cell line of interest.

1. Use dsRNA representing your target sequence (generated with the BLOCK-iT™ RNAi TOPO® Transcription Kit) in a reaction with the BLOCK-iT™ Dicer enzyme to generate d-siRNA.
2. Purify the d-siRNA using the purification reagents supplied in the kit. Quantitate the yield of purified d-siRNA obtained.
3. Transfect d-siRNA into the mammalian cell line of interest using Lipofectamine™ 2000 Reagent.

#### The RNAi Pathway and How Dicer Works

##### The RNAi Pathway

[0296] RNAi describes the phenomenon by which dsRNA induces potent and specific inhibition of eukaryotic gene expression via the degradation of

complementary messenger RNA (mRNA), and is functionally similar to the processes of post-transcriptional gene silencing (PTGS) or cosuppression in plants (Cogoni, C., *et al.*, *Antonie Van Leeuwenhoek* 65:205-209 (1994); Napoli, C., *et al.*, *Plant Cell* 2:279-289 (1990); Smith, C. J., *et al.*, *Mol. Gen. Genet.* 224:477-481 (1990); van der Krol, A. R., *et al.*, *Plant Cell* 2:291-299 (1990)) and quelling in fungi (Cogoni, C. and Macino, G., *Nature* 399:166-169 (1999); Cogoni, C. and Macino, G., *Proc. Natl. Acad. Sci. USA* 94:10233-10238 (1997); Romano, N. and Macino, G., *Mol. Microbiol.* 6:3343-3353 (1992)). In plants, the PTGS response is thought to occur as a natural defense against viral infection or transposon insertion (Anandalakshmi, R., *et al.*, *Proc. Natl. Acad. Sci. USA* 95:13079-13084 (1998); Jones, A. L., *et al.*, *EMBO J.* 17:6385-6393 (1998); Li, W.X. and Ding, S.W., *Curr. Opin. Biotechnol.* 12:150-154 (2001); Voinnet, O., *et al.*, *Proc. Natl. Acad. Sci. USA* 96:14147-14152 (1999)).

[0297] In eukaryotic organisms, dsRNA produced in vivo or introduced by pathogens is processed into 21-23 nucleotide double-stranded short interfering RNA duplexes (siRNA) by an enzyme called Dicer (Bernstein, E., *et al.*, *Nature* 409:363-366 (2001); Ketting, R.F., *et al.*, *Genes Dev.* 15:2654-2659 (2001)). The siRNA then incorporate into the RNA-induced silencing complex (RISC), a second enzyme complex that serves to target cellular transcripts complementary to the siRNA for specific cleavage and degradation (Hammond, S. M., *et al.*, *Nature* 404:293-296 (2000); Nykanen, A., *et al.*, *Cell* 107:309-321 (2001)).

[0298] For more information about the RNAi pathway and the mechanism of gene silencing, refer to recent reviews (Bosher, J. M. and Labouesse, M., *Nature Cell Biol.* 2:E31-E36 (2000); Hannon, G. J., *Nature* 418:244-251 (2002); Plasterk, R. H. A. and Ketting, R. F., *Genet. Dev.* 10:562-567 (2000); Zamore, P.D., *Biol.* 8:746-750 (2001)).

## Performing RNAi Analysis in Mammalian Cells

- [0299] A number of kits including the BLOCK-iT™ RNAi TOPO® Transcription Kit now exist to facilitate *in vitro* production of dsRNA that is targeted to a particular gene of interest. The dsRNA may be introduced directly into some invertebrate organisms or cell lines, where it functions to trigger the endogenous RNAi pathway resulting in inhibition of the target gene. Long dsRNA duplexes cannot be used directly for RNAi analysis in most somatic mammalian cell lines because introduction of long dsRNA into these cell lines induces a non-specific, interferon-mediated response, resulting in shutdown of translation and initiation of cellular apoptosis (Kaufman, R. J., *Proc. Natl. Acad. Sci. USA* 96:11693-11695 (1999)). To avoid triggering the interferon-mediated host cell response, dsRNA duplexes of less than 30 nucleotides must be introduced into cells (Stark, G. R., *et al.*, *Annu. Rev. Biochem.* 67:227-264 (1998)). For optimal results in gene knockdown studies, the size of the dsRNA duplexes (i.e. siRNA) introduced into mammalian cells is further limited to 21-23 nucleotides.

### Using the Kit for RNAi Analysis

- [0300] The BLOCK-iT™ Dicer RNAi Transfection Kit and the BLOCK-iT™ Complete Dicer RNAi Kit facilitate *in vitro* production of a complex pool of 21-23 nucleotide siRNA duplexes that is targeted to a particular gene of interest. The kits use a recombinant human Dicer enzyme (see below for more information) to cleave a long dsRNA substrate (produced with the BLOCK-iT™ RNAi TOPO® Transcription Kit) into a pool of 21-23 nucleotide d-siRNA that may be transfected into mammalian cells. Introduction of d-siRNA into the cells then triggers the endogenous RNAi pathway, resulting in inhibition of the target gene. For a diagram of the process, see Figure 6.

## BLOCK-iT™ Dicer Enzyme

[0301] BLOCK-iT™ Dicer is a recombinant human enzyme (Myers, J. W., *et al.*, *Nat. Biotechnol.* 21:324-328 (2003); Provost, P., *et al.*, *EMBO J.* 21:5864-5874 (2002)) that cleaves long dsRNA processively into 21-23 nucleotide d-siRNA duplexes with 2 nucleotide 3' overhangs. The Dicer enzyme is a member of the RNase III family of double-stranded RNA-specific endonucleases, and consists of an ATP-dependent RNA helicase domain, a Piwi/Argonaute/Zwille (PAZ) domain, two RNase III domains, and a dsRNA-binding domain (Bernstein, E., *et al.*, *Nature* 409:363-366 (2001); Zamore, P.D., *Biol.* 8:746-750 (2001)). In addition to its role in the generation of siRNA, Dicer is also involved in the processing of short temporal RNA (stRNA) (Hutvagner, G., *et al.*, *Science* 293:811-813 (2001); Ketting, R.F., *et al.*, *Genes Dev.* 15:2654-2659 (2001)) and microRNA (miRNA) (Carrington, J.C. and Ambros, V., *Science* 301:336-338 (2003)) from stable hairpin or stem-loop precursors.

## Experimental Outline

[0302] The table below outlines the desired steps when using the BLOCK-iT™ Dicer RNAi Kits to generate, purify, and transfect your d-siRNA of interest.

Step	Action
1	Produce dsRNA from your target gene.
2	Use the dsRNA in a reaction with the BLOCK-iT™ Dicer enzyme to generate d-siRNA.
3	Purify d-siRNA using the BLOCK-iT™ RNAi Purification Reagents.

4	Transfect purified d-siRNA into your mammalian cell line of interest using Lipofectamine™ 2000 Reagent.
5	Assay for inhibition of target gene expression using your method of choice.

## Methods

### Generating Double-Stranded RNA (dsRNA)

#### Introduction

[0303] Before you can use the BLOCK-iT™ Dicer Enzyme to produce short interfering RNA (siRNA), you should generate double-stranded RNA (dsRNA) substrate representing your target sequence of interest. Guidelines and recommendations to generate dsRNA are provided below.

[0304] For optimal, high-yield production of dsRNA, we recommend using the BLOCK-iT™ RNAi TOPO® Transcription Kit available from Invitrogen (Catalog no. K3500-01). The BLOCK-iT™ RNAi TOPO® Transcription Kit supplies the reagents necessary to generate T7 promoter-based DNA templates from any Taq-amplified PCR product, then use these templates in *in vitro* transcription reactions to generate sense and antisense RNA transcripts. The kit also includes reagents to enable purification and annealing of the RNA transcripts to produce high yields of dsRNA that are ready-to-use in the dicing reaction.

[0305] For detailed protocols and guidelines to generate dsRNA from your target gene sequence, refer to the BLOCK-iT™ RNAi TOPO® Transcription Kit manual. This manual is supplied with the BLOCK-iT™ Complete Dicer RNAi Kit.

## Choosing the Target Sequence

[0306] When performing RNAi analysis, your choice of target sequence can significantly affect the degree of gene knockdown observed. In addition, the size of the target sequence and the resulting dsRNA can affect the yields of dsRNA produced. Consider the following factors when choosing your target sequence.

Select a target sequence that covers a reasonable portion of the gene of interest and that does not contain regions of strong homology with other genes.

Limit the size of the target sequence. Although smaller or larger target sequences are possible, we recommend limiting the initial target sequence to a size range of 500 bp to 1 kb for the following reasons.

- (a) This balances the risk of including regions of strong homology between the target gene and other genes that could result in non-specific off-target effects during RNAi analysis with the benefits of using a more complex pool of siRNA.
- (b) When producing sense and antisense transcripts of the target template, the highest transcription efficiencies are obtained with transcripts in the 500 bp to 1 kb size range. Target templates outside this size range transcribe less efficiently, resulting in lower yields of dsRNA.
- (c) Double-stranded RNA that is under 1 kb in size is efficiently diced. Larger dsRNA substrates can be used but yields may decline as the size increases.

[0307] The BLOCK-iT™ Dicer RNAi Kits have been used successfully to knock down gene activity with dsRNA substrates ranging from 150 bp to 1.3 kb in size.

### Factors to Consider When Generating dsRNA

- [0308] If you are using your own method or another kit to produce dsRNA, consider the following factors when generating your dsRNA. These factors will influence the yields of d-siRNA produced from the dicing reaction.
- [0309] Amount of dsRNA desired for dicing: We use 60 µg of dsRNA in a typical 300 µl dicing reaction to recover 12-18 µg of d-siRNA after purification. This amount of d-siRNA is generally sufficient to transfect approximately 150 wells of cells plated in a 24-well format. You should have an idea of the scale and scope of your RNAi experiment to determine how much dsRNA you will need to dice.
- [0310] If you wish to dice less than 60 µg of dsRNA, you will need to scale down the dicing reaction proportionally.
- [0311] Concentration of dsRNA: The amount of dsRNA in a dicing reaction should not exceed half the reaction volume; therefore, the concentration of your dsRNA should be  $\geq 400$  ng/µl if you wish to dice 60 µg of dsRNA.
- [0312] Buffering of dsRNA: We recommend storing your dsRNA sample in a buffered solution containing 1 mM EDTA and no more than 100 mM salt (i.e. TE Buffer at pH 7-8 or 1X RNA Annealing Buffer). This helps to stabilize the dsRNA and provides the optimal environment for efficient cleavage by the Dicer Enzyme.
- [0313] If you have used the BLOCK-iT™ RNAi TOPO® Transcription Kit to produce dsRNA, your dsRNA sample will be in 1X RNA Annealing Buffer (10 mM Tris-HCl, 20 mM NaCl, 1 mM EDTA, pH 8.0).
- [0314] The quality of your dsRNA: To obtain the highest yields of d-siRNA, we recommend using purified dsRNA in the dicing reaction.
- [0315] Once you have generated your purified dsRNA, we recommend saving an aliquot of the dsRNA for future gel analysis. We generally use agarose or polyacrylamide gel electrophoresis to assess the success of the dicing reaction by comparing an aliquot of the dicing reaction to an aliquot of the dsRNA substrate.

## Performing the Dicing Reaction

- [0316] Once you have produced your target dsRNA, you will perform an in vitro dicing reaction using the reagents supplied in the BLOCK-iT™ Dicer Enzyme Kit (Box 1) to generate d-siRNA duplexes of 21-23 nucleotides in size.

## BLOCK-iT™ Dicer Enzyme Activity

- [0317] One unit of BLOCK-iT™ Dicer Enzyme cleaves 1 µg of dsRNA in 16 hours at 37°C. Note that the Dicer enzyme does not cleave dsRNA to d-siRNA with 100% efficiency, i.e. dicing 1 µg of dsRNA does not generate 1 µg of d-siRNA. Under these optimal reaction conditions, the Dicer enzyme cleaves dsRNA to d-siRNA with an efficiency of approximately 25-35%. For example, dicing 60 µg of dsRNA in a 300 µl dicing reaction typically yields 12-18 µg of d-siRNA following purification.
- [0318] For best results, we recommend following the dicing procedure exactly as described as the reaction conditions have been optimized to provide the highest mass yield of d-siRNA under the most efficient dicing conditions.
- [0319] It is possible to use more than 60 µg of dsRNA in a 300 µl dicing reaction; however, the BLOCK-iT™ Dicer Enzyme becomes less efficient under these conditions. Although you may generate a higher mass yield of d-siRNA, the % yield of d-siRNA will decrease.
- [0320] Do not increase the amount of BLOCK-iT™ Dicer Enzyme used in the dicing reaction (to greater than 60 units in a 300 µl reaction) or increase the length of the dicing reaction (to greater than 18 hours). Under either of these conditions, the BLOCK-iT™ Dicer Enzyme can bind to d-siRNA and cleave the 21-23 nt duplexes into smaller products, resulting in lower yields of d-siRNA.



#### Amount of dsRNA to Use

- [0321] For a typical 300 µl dicing reaction, you will need 60 µg of target dsRNA. If you want to dice less than 60 µg of dsRNA, scale down the entire reaction proportionally.
- [0322] The total volume of dsRNA added should not exceed half the volume of the reaction. Thus, for best results, make sure that the starting concentration of your dsRNA is  $\geq 400$  ng/µl.

#### Positive Control

- [0323] If you are using the BLOCK-iT™ Complete Dicer RNAi Kit, and have performed all of the recommended control reactions using the control reagents supplied in the BLOCK-iT™ RNAi TOPO® Transcription portion of the kit, you should have purified dsRNA representing a 1 kb portion of the lacZ gene. We recommend setting up a separate dicing and purification reaction using the control lacZ dsRNA. You can then co-transfect the resulting purified lacZ d-siRNA and the pcDNA™1.2/V5-GW/lacZ control plasmid supplied with the kit into your mammalian cell line as a positive control for the RNAi response in that cell line. Alternatively, you may use the lacZ d-siRNA as a negative control for non-specific, off-target effects in your cell line.
- [0324] When performing the dicing reaction and subsequent purification of d-siRNA, take precautions to avoid RNase contamination.
- [0325] Use RNase-free sterile pipette tips and supplies for all manipulations.
- [0326] Use DEPC-treated solutions as necessary.
- [0327] Wear gloves when handling reagents and solutions, and when performing reactions.

#### Materials Needed

- [0328] Have the following reagents on hand before beginning:  
Purified dsRNA ( $> 400$  ng/µl in 1X RNA Annealing Buffer or TE Buffer, pH 7-8)

BLOCK-iT™ Dicer Enzyme (1 U/μl; supplied with the kit, Box 1;  
keep at -20°C until immediately before use)

10X Dicer Buffer (supplied with the kit, Box 1)

RNase-Free Water (supplied with the kit, Box 1)

50X Dicer Stop Buffer (supplied with the kit, Box 1)

#### Dicing Procedure

[0329] Follow the procedure below to perform the dicing reaction. Make sure that the volume of dsRNA added does not exceed half the volume of the reaction (i.e. ≤ 150 μl).

1. Set up a 300 μl dicing reaction on ice using the following reagents in the order shown.

Reagent	Sample
10X Dicer Buffer	30 μl
RNase-Free Water	up to 210 μl
Purified dsRNA (60 μg)	1-150 μl
BLOCK-iT™ Dicer Enzyme (1 U/μl)	60 μl
Total volume	300 μl

2. Mix reaction gently and incubate for 14-18 hours at 37°C.

Do not incubate the reaction for longer than 18 hours as this may result in a lower yield of d-siRNA due to cleavage of d-siRNA by the Dicer enzyme.

3. Add 6 μl of 50X Dicer Stop Solution to the reaction.

4. Check the integrity of your d-siRNA, if desired.

5. Proceed to purify the d-siRNA (see Purifying Diced siRNA (d-siRNA),) or store the dicing reaction overnight at -20°C.

#### Checking the Integrity of d-siRNA

[0330] You may verify the integrity of your d-siRNA using polyacrylamide or agarose gel electrophoresis, if desired. We suggest running an aliquot of your

dicing reaction (0.5-1  $\mu$ l of a 300  $\mu$ l reaction; equivalent to 100-200 ng of dsRNA) on the appropriate gel and comparing it to an aliquot of your starting dsRNA. Be sure to include an appropriate molecular weight standard. We generally use the following gels and molecular weight standard:

Agarose gel: 4% E-Gel® (Invitrogen, Catalog no. G5000-04)

Polyacrylamide gel: 20% Novex® TBE Gel (Invitrogen, Catalog no. EC63152BOX)

Molecular weight standard: 10 bp DNA Ladder (Invitrogen, Catalog no. 10821-015)

[0331] When analyzing an aliquot of the dicing reaction by gel electrophoresis, we generally see the following:

A predominant band of approximately 21-23 nt representing the d-siRNA.

4% E-Gel®: A high molecular weight smear representing uncleaved dsRNA and partially cleaved products. Generally, this band does not resolve well on an agarose gel and runs close to the well.

Novex® 20% TBE Gel: A high molecular weight band and a smear representing uncleaved dsRNA and partially cleaved products. The dsRNA band generally resolves better on a polyacrylamide gel.

[0332] If the band representing d-siRNA is weak or if you do not see a band, see Troubleshooting for tips to troubleshoot your dicing reaction.

#### Example of Expected Results

[0333] In this experiment, purified dsRNA representing a 1 kb region of the lacZ gene was generated following the recommended protocols and using the reagents supplied in the BLOCK-iT™ RNAi TOPO® Transcription Kit. The lacZ dsRNA was diced using the procedure outlined below. Aliquots of the dicing reaction (equivalent to 200 ng of dsRNA) and the initial dsRNA substrate were analyzed on a 4% E-Gel®.

[0334] Results are shown in Figure 7: A prominent band representing d-siRNA of the expected size is clearly visible in the dicing reaction sample

(lane 3). This band is not visible in the initial dsRNA substrate sample (lane 2). Lane 1. 10 bp DNA Ladder. Lane 2. 200 ng purified *lacZ* dsRNA. Lane 3. 200 ng *lacZ* dicing reaction.

## Purifying Diced siRNA (d-siRNA)

### Introduction

[0335] This section provides guidelines and instructions to purify the d-siRNA produced in the dicing reaction. Use the BLOCK-iT™ RNAi Purification reagents (Box 2) supplied with the kit.

[0336] Before proceeding to transfection, note that you should purify the d-siRNA produced in the dicing reaction to remove contaminating long dsRNA duplexes. Transfection of unpurified d-siRNA can trigger the interferon-mediated response and cause host cell shutdown and cellular apoptosis. When purifying d-siRNA, follow the purification procedure provided below exactly as instructed. This procedure is optimized to allow removal of contaminating long dsRNA and recovery of high yields of d-siRNA.

### Experimental Outline

[0337] To purify d-siRNA, you will:

1. Add RNA Binding Buffer and isopropanol to the dicing reaction to denature the proteins and to enable the contaminating dsRNA to bind to the column.
2. Add half the volume of the sample to an RNA spin cartridge. The dsRNA binds to the silica-based membrane in the cartridge, and the d-siRNA and denatured proteins flow through the cartridge. Save the flow-through.
3. Transfer the RNA spin cartridge to an siRNA Collection Tube and add the remaining sample to the RNA spin cartridge. Repeat Step 2. Save the flow-through.

4. Pool the flow-throughs from Step 2 and Step 3 in the siRNA Collection Tube and add isopropanol to the sample to enable the d-siRNA to bind to the column.
5. Add the sample to a second RNA spin cartridge. The d-siRNA bind to the membrane in the cartridge.
6. Wash the membrane-bound d-siRNA to eliminate residual RNA Binding Buffer, isopropanol, and any remaining impurities.
7. Elute the d-siRNA from the RNA spin cartridge with water.
8. Add 50X RNA Annealing Buffer to the eluted d-siRNA to stabilize the d-siRNA for storage.

[0338] For an illustration of the d-siRNA purification process, see Figure 8.

#### Advance Preparation

[0339] Before using the BLOCK-iT™ RNA Purification reagents for the first time, add 10 ml of 100% ethanol to the entire amount of 5X RNA Wash Buffer to obtain a 1X RNA Wash Buffer (total volume = 12.5 ml). Place a check in the box on the 5X RNA Wash Buffer label to indicate that the ethanol was added. Store the 1X RNA Wash Buffer at room temperature.

[0340] The RNA Binding Buffer contains guanidine isothiocyanate. This chemical is harmful if it comes in contact with the skin or is inhaled or swallowed. Always wear a laboratory coat, disposable gloves, and goggles when handling solutions containing this chemical.

[0341] Do not add bleach or acidic solutions directly to solutions containing guanidine isothiocyanate or sample preparation waste. Guanidine isothiocyanate forms reactive compounds and toxic gases when mixed with bleach or acids.

#### Materials Needed

[0342] Have the following materials on hand before beginning:  
Dicing reaction (from Step 5)  
RNA Binding Buffer (supplied with the kit, Box 2)

$\beta$ -mercaptoethanol

Isopropanol

RNA Spin Cartridges (supplied with the kit, Box 2; two for each sample)

siRNA Collection Tube (supplied with the kit, Box 2)

1X RNA Wash Buffer (see Advance Preparation, above)

RNase-Free Water (supplied with the kit, Box 2)

RNA Recovery Tube (supplied with the kit, Box 2)

50X RNA Annealing Buffer (supplied with the kit, Box 2)

RNase-free supplies

#### d-siRNA Purification Procedure

[0343] Use this procedure to purify d-siRNA produced from dicing 60  $\mu$ g of dsRNA in a 300  $\mu$ l reaction volume (see Step 5). If you have digested < 60  $\mu$ g of dsRNA and have scaled down the volume of your dicing reaction, scale down the volume of your purification reagents proportionally. For example, if you have digested 30  $\mu$ g of dsRNA in a 150  $\mu$ l dicing reaction, scale down the volume of purification reagents used by half.

[0344] Before beginning, remove the amount of RNA Binding Buffer needed and add  $\beta$ -mercaptoethanol to a final concentration of 1% (v/v). Use fresh and discard any unused solution.

1. To each dicing reaction (~300  $\mu$ l volume), add 300  $\mu$ l of RNA Binding Buffer containing 1% (v/v)  $\beta$ -mercaptoethanol followed by 300  $\mu$ l of isopropanol to obtain a final volume of 900  $\mu$ l. Mix well by pipetting up and down 5 times.
2. Apply half of the sample (~450  $\mu$ l) to the RNA Spin Cartridge. Centrifuge at 14,000 x g for 15 seconds at room temperature.
3. Transfer the RNA spin cartridge to an siRNA Collection Tube. Save the flow-through containing d-siRNA from Step 2.

4. Apply the remaining half of the sample (~450  $\mu$ l) to the RNA Spin Cartridge. Centrifuge at 14,000 x g for 2 minutes at room temperature.
5. Remove the RNA Spin Cartridge from the siRNA Collection Tube and discard. Save the flow-through containing d-siRNA.
6. Transfer the flow-through from Step 2 (~450  $\mu$ l) to the siRNA Collection Tube containing the flow-through from Step 4 (~450  $\mu$ l) to obtain a final volume of ~900  $\mu$ l. Add 600  $\mu$ l of isopropanol to the sample to obtain a final volume of 1.5 ml. Mix well by pipetting up and down.
7. Apply one-third of the sample (~500  $\mu$ l) to a new RNA Spin Cartridge. Centrifuge at 14,000 x g for 15 seconds at room temperature. Discard the flow-through.
8. Repeat Step 7 twice, applying one-third of the remaining sample (~500  $\mu$ l) to the RNA Spin Cartridge each time.
9. Add 500  $\mu$ l of 1X RNA Wash Buffer to the RNA Spin Cartridge containing bound d-siRNA. Centrifuge at 14,000 x g for 15 seconds at room temperature. Discard the flow-through.
10. Repeat the wash step (Step 9).
11. Centrifuge the RNA Spin Cartridge at 14,000 x g for 1 minute at room temperature to remove residual 1X RNA Wash Buffer from the cartridge and to dry the membrane.
12. Remove the RNA Spin Cartridge from the Wash Tube, and place it in an RNA Recovery Tube.
13. Add 30  $\mu$ l of RNase-Free Water to the RNA Spin Cartridge. Let stand at room temperature for 1 minute, then centrifuge the RNA Spin Cartridge at 14,000 x g for 2 minutes at room temperature to elute the d-siRNA. Proceed to Step 14.
14. Add 30  $\mu$ l of RNase-Free Water to the RNA Spin Cartridge and repeat Step 13, eluting the d-siRNA into the same RNA Recovery Tube. The total volume of eluted d-siRNA is 60  $\mu$ l.

15. Add 1.2  $\mu$ l of the 50X RNA Annealing Buffer to the eluted d-siRNA to obtain a final concentration of 1X RNA Annealing Buffer. Adding RNA Annealing Buffer to the sample increases the stability of the d-siRNA.
  16. Proceed to quantitate the concentration of your purified d-siRNA (see Determining the Purity and Concentration of d-siRNA, below).
  17. Store the purified d-siRNA at -80°C. Depending on the amount of d-siRNA produced and your downstream application, you may want to aliquot the d-siRNA before storage at -80°C.
- [0345] When using the d-siRNA, avoid repeated freezing and thawing as d-siRNA can degrade with each freeze/thaw cycle.

#### Determining the Purity and Concentration of d-siRNA

- [0346] Use the procedure below to determine the purity and concentration of your purified d-siRNA.
1. Dilute an aliquot of the purified d-siRNA 20-fold into 1X RNA Annealing Buffer in a total volume appropriate for your quartz cuvettes and spectrophotometer.
  2. Measure OD at A260 and A280 in a spectrophotometer. Blank the sample against 1X RNA Annealing Buffer.
  3. Calculate the concentration of the d-siRNA by using the following equation:  
$$\text{d-siRNA concentration } (\mu\text{g/ml}) = \text{A260} \times \text{Dilution factor (20)} \times 40 \mu\text{g/ml}$$
  4. Calculate the yield of the d-siRNA by using the following equation:  
$$\text{d-siRNA yield } (\mu\text{g}) = \text{d-siRNA concentration } (\mu\text{g/ml}) \times \text{vol. of d-siRNA (ml)}$$
  5. Evaluate the purity of the purified d-siRNA by determining the A260/A280 ratio. For optimal purity, the A260/A280 ratio should range from 1.9-2.2.



### Verifying the Quality of Your d-siRNA

[0347] You may verify the quality of your purified d-siRNA using polyacrylamide or agarose gel electrophoresis, if desired. We suggest running a small aliquot of your purified d-siRNA (0.5-1  $\mu$ l) on the appropriate gel and comparing it to an aliquot of your dicing reaction (equivalent to 100-200 ng of dsRNA). Be sure to include an appropriate molecular weight standard. For recommended gels and a molecular weight standard, we generally use the same gels and molecular weight standard that we use to analyze the quality of the dicing reaction.

[0348] If the band representing purified d-siRNA is weak or if you do not see a band, see Troubleshooting for tips to purify your d-siRNA.

### Example of Expected Results

[0349] In this experiment, the *lacZ* d-siRNA generated in the dicing reaction depicted above were purified using the procedure described above. Aliquots of the purified *lacZ* d-siRNA (80 ng) and the *lacZ* dicing reaction (equivalent to 200 ng of dsRNA) were analyzed on a 4% E-Gel®.

[0350] Results are demonstrated in Figure 9.: A prominent band representing purified d-siRNA of the expected size is clearly visible in lane 3. No contaminating dsRNA or other high molecular weight products remain in the purified d-siRNA sample. Lane 1. 10 bp DNA Ladder, Lane 2. 200 ng *lacZ* dicing reaction, Lane 3. 80 ng purified *lacZ* d-siRNA.

[0351] The typical yield of d-siRNA obtained from dicing 60  $\mu$ g of dsRNA (500 bp to 1 kb in size) in a 300  $\mu$ l dicing reaction ranges from 12-18  $\mu$ g, with a concentration of 200-300 ng/ $\mu$ l. Note that yields may vary depending on the size and quality of the dsRNA.

## Transfecting Cells

### Introduction

[0352] Once you have purified your d-siRNA, you may perform RNAi analysis by transfecting the d-siRNA into the mammalian cell line of interest, and assaying for inhibition of expression from your target gene. This section provides general guidelines and protocols to transfect your purified d-siRNA into mammalian cells using the Lipofectamine™ 2000 Reagent (Box 3) supplied with the kit. Suggested transfection conditions are provided as a starting point. You will need to optimize transfection conditions to obtain the best results for your target gene and mammalian cell line.

[0353] You must transfect mammalian cells with purified d-siRNA. Note that transfecting cells with unpurified d-siRNA containing contaminating long dsRNA (i.e. with material directly taken from the dicing reaction) can trigger the interferon-mediated cellular response, resulting in host cell shutdown and cellular apoptosis.

### Factors Affecting Gene Knockdown Levels

[0354] A number of factors can influence the degree to which expression of your gene of interest is reduced (i.e. gene knockdown) in an RNAi experiment including:

- Transfection efficiency

- Transcription rate of the target gene of interest

- Stability of the target protein

- Growth characteristics of your mammalian cell line

[0355] Take these factors into account when designing your transfection and RNAi experiments.

### Lipofectamine™ 2000 Reagent

[0356] The Lipofectamine™ 2000 Reagent supplied with the kit is a cationic lipid-based formulation suitable for the transfection of nucleic acids including d-siRNA and siRNA into eukaryotic cells (Ciccarone, V., *et al.*, *Focus* 21:54-55 (1999); Gitlin, L., *et al.*, *Nature* 418:430-434 (2002); Yu, J.Y., *et al.*, *Proc. Nat. Acad. Sci. USA* 99:6047-6052 (2002)). Using Lipofectamine™ 2000 to transfect d-siRNA into eukaryotic cells offers the following advantages:

Provides the highest transfection efficiency in many cell types

Is the most widely used transfection reagent for delivery of d-siRNA or siRNA into eukaryotic cells (Gitlin, L., *et al.*, *Nature* 418:430-434 (2002); Yu, J.Y., *et al.*, *Proc. Nat. Acad. Sci. USA* 99:6047-6052 (2002))

d-siRNA-Lipofectamine™ 2000 complexes can be added directly to cells in culture medium in the presence of serum.

Removal of complexes, medium change, or medium addition following transfection are not required, although complexes can be removed after 4-6 hours without loss of activity.

[0357] Lipofectamine™ 2000 is also available separately from Invitrogen.

### Important Guidelines

[0358] Follow these guidelines when transfecting siRNA into mammalian cells using Lipofectamine™ 2000:

1. Cell density: For optimal results, we recommend plating cells such that they will be 30-50% confluent at the time of transfection. Gene knockdown levels are generally assayed 24-72 hours following transfection. Transfecting cells at a lower density allows a longer interval between transfection and assay time, and minimizes the loss of cell viability due to cell overgrowth. Depending on the nature of the target gene, higher or lower cell densities may be suitable with optimization of conditions.

2. For optimal results, use Opti-MEM® I Reduced Serum Medium (Invitrogen, Catalog no. 31985-062) to dilute Lipofectamine™ 2000 and d-siRNA prior to complex formation.
3. Do not include antibiotics in media used during transfection as this will reduce transfection efficiency and cause cell death.

#### Materials to Have on Hand

[0359] Have the following materials on hand before beginning:

Mammalian cell line of interest (make sure that cells are healthy and greater than 90% viable before transfection)

Purified d-siRNA of interest ( $\geq 40$  ng/ $\mu$ l)

If you have diced 60  $\mu$ g of dsRNA, the typical yield of d-siRNA obtained after purification is 12-18  $\mu$ g at a concentration of 200-300 ng/ $\mu$ l)

Positive control, if desired (see below)

Lipofectamine™ 2000 Reagent (supplied with the kit; store at +4°C until use)

Opti-MEM® I Reduced Serum Medium (Invitrogen, Catalog no. 31985-062; pre-warmed)

Sterile tissue culture plates and other tissue culture supplies

#### Positive Control

[0360] If you are using the BLOCK-iT™ Complete Dicer RNAi Kit, and have diced the control lacZ dsRNA, two options exist to use the resulting purified lacZ d-siRNA for RNAi analysis:

1. Use the lacZ d-siRNA as a negative control for non-specific off-target effects.
2. Use the lacZ d-siRNA as a positive control to assess the RNAi response in your cell line by co-transfecting the lacZ d-siRNA and the pcDNA™1.2/V5-GW/lacZ reporter plasmid supplied with the kit into your mammalian cells using Lipofectamine™ 2000. Assay for

knockdown of  $\beta$ -galactosidase expression 24 hours post-transfection using Western blot analysis or activity assay.

- [0361] Transfection conditions (i.e. cell density and reagent amounts) vary slightly when d-siRNA and plasmid DNA are co-transfected into mammalian cells. For details, see Co-transfecting d-siRNA and Plasmid DNA.

#### Transfection Procedure

- [0362] Use this procedure to transfect mammalian cells using Lipofectamine™ 2000. Refer to the table in Recommended Reagent Amounts and Volumes, below for the appropriate reagent amounts and volumes to add for different tissue culture formats. Use the recommended Lipofectamine™ 2000 amounts as a starting point for your experiments, and optimize conditions for your cell line and d-siRNA.

1. One day before transfection, plate cells in the appropriate amount of growth medium without antibiotics such that they will be 30-50% confluent at the time of transfection.
2. For each transfection sample, prepare d-siRNA:Lipofectamine™ 2000 complexes as follows:
  - (a) Dilute d-siRNA in the appropriate amount of Opti-MEM® I Reduced Serum Medium without serum. Mix gently.
  - (b) Mix Lipofectamine™ 2000 gently before use, then dilute the appropriate amount in Opti-MEM® I Reduced Serum Medium. Mix gently and incubate for 5 minutes at room temperature. Combine the diluted Lipofectamine™ 2000 with the diluted d-siRNA within 30 minutes. Longer incubation times may decrease activity.
  - (c) After the 5 minute incubation, combine the diluted d-siRNA with the diluted Lipofectamine™ 2000. Mix gently and incubate for 20 minutes at room temperature to allow the d-siRNA:Lipofectamine™ 2000 complexes to form. The solution may appear cloudy, but this will not inhibit transfection.

3. Add the d-siRNA:Lipofectamine™ 2000 complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.
4. Incubate the cells at 37°C in a CO<sub>2</sub> incubator for 24-96 hours as appropriate until they are ready to assay for gene knockdown. It is not necessary to remove the complexes or change the medium; however, growth medium may be replaced after 4-6 hours without loss of transfection activity.

#### Recommended Reagent Amounts and Volumes

[0363] The table below lists the recommended reagent amounts and volumes to use to transfect cells in various tissue culture formats. Use the recommended amounts of d-siRNA (see column 4) and Lipofectamine™ 2000 (see column 6) as a starting point for your experiments, and optimize conditions for your cell line and target gene. With automated, high-throughput systems, larger complexing volumes are recommended for transfections in 96-well plates.

Culture Vessel	Relative Surface Area (vs. 24-well)	Volume of Plating Medium	d-siRNA (µg) and Dilution Volume (µl)	d-siRNA Amounts (µl) for Optimization	Lipofectamine™ 2000 (µl) and Dilution Volume (µl)	Lipofectamine™ 2000 Amounts (µl) for Optimization
96-well	0.2	100 µl	20 ng in 25 µl	5-50 ng	0.6 µl in 25 µl	0.2-1.0 µl
24-well	1	500 µl	50 ng in 50 µl	20-200 ng	1 µl in 50 µl	0.5-1.5 µl
6-well	5	2 ml	250 ng in 250 µl	100 ng-1 µg	5 µl in 250 µl	2.5-6 µl

#### Optimizing Transfection

[0364] To obtain the highest transfection efficiency and low non-specific effects, optimize transfection conditions by varying the cell density (from 30-50% confluence) and the amounts of d-siRNA (see column 5) and

Lipofectamine™ 2000 (see column 7) as suggested in the table above. For cell lines that are particularly sensitive to transfection-mediated cytotoxicity (e.g. HeLa, HT1080), use the lower amounts of Lipofectamine™ 2000 suggested in the table above.

#### What You Should See

- [0365] When performing RNAi experiments using d-siRNA, we generally observe inhibition of the gene of interest within 24 to 96 hours after transfection. The degree of gene knockdown depends on the time of assay, stability of the protein of interest, and on the other factors. Note that 100% gene knockdown is generally not observed, but > 95% is possible with optimized conditions.

#### Co-transfecting d-siRNA and Plasmid DNA

- [0366] If you are using the lacZ d-siRNA as a positive control to assess the RNAi response in your cell line, you will co-transfect the lacZ d-siRNA and the pcDNA™ 1.2/V5-GW/lacZ reporter plasmid into the mammalian cell line and assay for inhibition of  $\beta$ -galactosidase expression after 24 hours. When co-transfecting d-siRNA and plasmid DNA, follow the procedure on the previous page with the following exceptions:

Plate cells such that they will be 90% confluent at the time of transfection.

Refer to the table below for the recommended amount of d-siRNA (see column 3) and plasmid DNA (see column 4) to transfect in a particular tissue culture format.

We generally transfect twice the mass of plasmid DNA as d-siRNA.

Use the recommended Lipofectamine™ 2000 amounts in the table below (see column 6) as a starting point, and optimize conditions for your cell line if desired. To optimize conditions, vary the amount of Lipofectamine™ 2000 as suggested in the table below (see column 7).

Culture Vessel	Volume of Plating Medium	d-siRNA (μg)	Plasmid DNA (μg)	Nucleic Acid Dilution Volume	Lipofect-amine™ 2000 (μl) and Dilution Volume (μl)	Lipofect-amine™ 2000 Amounts (μl) for Optimization
96-well	100 μl	20 ng	40 ng	25 μl	0.6 μl in 25 μl	0.2-1.0 μl
24-well	500 μl	50 ng	100 ng	50 μl	2 μl in 50 μl	0.5-2.0 μl
6-well	2 ml	250 ng	500 ng	250 μl	10 μl in 250 μl	2.5-10 μl

#### Assaying for β-galactosidase Expression

[0367] If you perform RNAi analysis using the control lacZ d-siRNA, you may assay for β-galactosidase expression and knockdown by Western blot analysis or activity assay using cell-free lysates (Miller, J. H., *Experiments in Molecular Genetics* (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1972)). Invitrogen offers the β-gal Antiserum (Catalog no. R901-25) and the β-Gal Assay Kit (Catalog no. K1455-01) for fast and easy detection of β-galactosidase expression.

[0368] The β-galactosidase protein expressed from the pcDNA™1.2/V5-GW/lacZ control plasmid is fused to a V5 epitope and is approximately 119 kDa in size. If you are performing Western blot analysis, you may also use the Anti V5 Antibodies available from Invitrogen (e.g. Anti-V5-HRP Antibody; Catalog no. R961-25 or Anti-V5-AP Antibody, Catalog no. R962-25) for detection.

#### Examples of Expected Results

##### Introduction

[0369] This section provides some examples of results obtained from RNAi experiments performed with d-siRNA generated using the BLOCK-iT™ Complete Dicer RNAi Kit. The first example depicts knockdown of expression of a reporter gene, and the second example depicts knockdown of expression of the endogenous lamin A/C gene.



#### Example of Expected Results: Knockdown of a Reporter Gene

[0370] In this experiment, d-siRNA targeting two reporter genes (i.e. luciferase and lacZ) and an endogenous gene (i.e. lamin A/C) was generated following the recommended protocols and using the reagents supplied in the BLOCK-iT™ Complete Dicer RNAi Kit.

[0371] GripTite™ 293 MSR cells (Invitrogen, Catalog no. R795-07) were grown to 90% confluence. Individual wells in a 24-well plate were transfected using Lipofectamine™ 2000 Reagent with 100 ng each of lacZ and luciferase-containing reporter plasmids. In some wells, the reporter plasmids were co-transfected with 50 ng of purified lacZ, luciferase, or lamin A/C d-siRNA. Cell lysates were prepared 24 hours after transfection and assayed for luciferase and  $\beta$ -galactosidase activity. Activities were normalized to those of the reporter plasmids alone.

[0372] Results are shown in Figure 10: Potent and specific inhibition is evident from luciferase and lacZ-derived d-siRNA. Note that in this experiment, lamin A/C d-siRNA serves as a negative control and does not inhibit luciferase or  $\beta$ -galactosidase expression.

[0373] Introduction of d-siRNA into mammalian cells can, in some cases lead to a slight induction of gene expression, as is observed with  $\beta$ -galactosidase and luciferase expression upon transfection of lamin d-siRNA.

#### Example of Expected Results: Knockdown of an Endogenous Gene

[0374] In this experiment, dsRNA representing a 1 kb region of the lamin A/C gene and the luciferase gene were produced following the recommended protocols and using reagents supplied in the BLOCK-iT™ RNAi TOPO® Transcription Kit. The target sequences chosen for the lamin A/C and luciferase genes were as described by (Elbashir, S. M., *et al.*, *Nature* 411:494-498 (2001)). The resulting dsRNA were used as substrates to generate lamin A/C and luciferase d-siRNA following the recommended protocols and using the reagents supplied in the BLOCK-iT™ Complete Dicer RNAi Kit.

[0375] 50 ng each of lamin A/C and luciferase d-siRNA as well as 4 pmoles each (about 50 ng) of synthetic lamin A/C and luciferase siRNA (21 nucleotide duplexes) were transfected into A549 (human lung carcinoma) cells plated in a 24-well plate using Lipofectamine™ 2000. Cell lysates were prepared 48 hours post-transfection and analyzed by Western blot using an Anti-Lamin A/C Antibody (1:1000 dilution, BD Biosciences, Catalog no. 612162) and an Anti- $\beta$ -Actin Antibody (1:5000 dilution, Abcam, Catalog no. ab6276).

[0376] Results are shown in Figure 11: Only the lamin A/C-specific d-siRNA (lane 2) and siRNA (lane 4) were able to inhibit expression of the lamin A/C gene, while no lamin A/C gene knockdown was observed with the luciferase d-siRNA (lane 3) or siRNA (lane 5). In addition, the degree of lamin A/C gene blocking achieved using the lamin A/C d-siRNA was similar to that achieved with the well-characterized, chemically-synthesized siRNA. Lane 1. Mock transfection, Lane 2. 50 ng lamin A/C d-siRNA, Lane 3. 50 ng luciferase d-siRNA, Lane 4. 4 pmol lamin A/C siRNA, Lane 5. 4 pmol luciferase siRNA.

#### Troubleshooting

[0377] Use the information in this section to troubleshoot your dicing, purification, and transfection experiments.

## Dicing Reaction

[0378] The table below lists some potential problems and possible solutions that may help you troubleshoot the dicing reaction.

Problem	Reason	Solution
Weak band representing d-siRNA observed on a poly-acrylamide or agarose gel (i.e. low yield of d-siRNA)	Poor quality dsRNA	<ul style="list-style-type: none"> <li>• Generate dsRNA using the BLOCK-iT™ RNAi TOPO® Transcription Kit (refer to the BLOCK-iT™ RNAi TOPO® Transcription Kit manual for instructions).</li> <li>• Verify the concentration of your dsRNA.</li> </ul>
	Didn't use enough dsRNA in the dicing reaction	<ul style="list-style-type: none"> <li>• Use 60 µg of dsRNA in a 300 µl dicing reaction. If you are dicing less dsRNA, scale down the entire dicing reaction proportionally.</li> <li>• Make sure that the amount of dsRNA added does not exceed half the reaction volume (i.e. concentration of initial dsRNA substrate &gt;400 ng/µl).</li> </ul>
	dsRNA was degraded	<ul style="list-style-type: none"> <li>• Make sure that the dsRNA sample is in a buffer containing 1 mM EDTA (i.e. TE Buffer, pH 7-8 or 1X RNA Annealing Buffer).</li> <li>• Avoid repeated freeze/thaw cycles. Aliquot the dsRNA and store at -80°C.</li> </ul>
	Incubated the dicing reaction for longer than 18 hours	Do not incubate the dicing reaction for longer than 18 hours.

Problem	Reason	Solution
	Incubated the dicing reaction for less than 14 hours	Incubate the dicing reaction at 37°C for 14-18 hours.
Smear with molecular weight < 21 nt observed on a poly-acrylamide gel	Used too much BLOCK-iT™ Dicer Enzyme in the dicing reaction	Follow the recommended procedure to set up the dicing reaction. Do not use more than 60 units of BLOCK-iT™ Dicer Enzyme in a 300 µl reaction.
	Incubated the dicing reaction for longer than 18 hours	Do not incubate the dicing reaction for longer than 18 hours.
	Sample contaminated with RNase	<ul style="list-style-type: none"> <li>• Use RNase-free supplies and solutions.</li> <li>• Wear gloves when handling reagents and setting up the dicing reaction.</li> </ul>
No d-siRNA produced	dsRNA was degraded	<ul style="list-style-type: none"> <li>• Make sure that the dsRNA sample is in a buffer containing 1 mM EDTA (i.e. TE Buffer, pH 7-8 or 1X RNA Annealing Buffer).</li> <li>• Avoid repeated freeze/thaw cycles. Aliquot the dsRNA and store at -80°C.</li> </ul>
	Sample was contaminated with RNase	<ul style="list-style-type: none"> <li>• Use RNase-free supplies and solutions.</li> <li>• Wear gloves when handling reagents and setting</li> </ul>

Problem	Reason	Solution
	ssRNA used as substrate	If you have used to the BLOCK-iT™ RNAi TOPO® Transcription Kit to generate sense and antisense ssRNA, you should anneal the ssRNA to generate dsRNA prior to dicing.

### Purifying d-siRNA

[0379] The table below lists some potential problems and possible solutions that may help you troubleshoot the purification procedure.

Problem	Reason	Solution
Low yield of purified d-siRNA obtained	Eluted d-siRNA from the RNA Spin Cartridge using TE Buffer	Elute d-siRNA from the RNA Spin Cartridge using water.
	Concentration of d-siRNA incorrectly determined <ul style="list-style-type: none"> <li>• Sample diluted into water for spectrophotometry</li> <li>• Sample blanked against water</li> </ul>	<ul style="list-style-type: none"> <li>• Dilute sample in 1X RNA Annealing Buffer for spectrophotometry.</li> <li>• Blank sample against 1X RNA Annealing Buffer.</li> </ul>
No d-siRNA obtained	Forgot to add ethanol to the 5X RNA Wash Buffer	Add 10 ml of ethanol to the 5X RNA Wash Buffer (2.5 ml) to obtain a 1X RNA Wash Buffer.

Problem	Reason	Solution
	Forgot to add isopropanol to the combined flow-throughs from the first RNA Spin Cartridge	You should add isopropanol to the combined flow-throughs from the first RNA Spin Cartridge to enable the d-siRNA to bind to the second RNA Spin Cartridge.
	Forgot to keep flow-throughs from the first RNA Spin Cartridge	Keep the flow-throughs from the first RNA Spin Cartridge (Steps 3 and 5). The flow-throughs contain the d-siRNA.
dsRNA present in purified d-siRNA sample	Forgot to add isopropanol to the dicing reaction	You should add RNA Binding Buffer containing 1% (v/v) $\beta$ -mercaptoethanol and isopropanol to the dicing reaction to denature the proteins and enable the dsRNA to bind the first RNA Spin Cartridge.
	Added the mixture containing the flow-through and isopropanol from the first RNA Spin Cartridge (Step 6) back onto the first RNA Spin Cartridge	You should add the mixture containing the flow-through and isopropanol from the first RNA Spin Cartridge (Step 6) to a second RNA Spin Cartridge as the first RNA Spin Cartridge contains bound dsRNA.
A260/A280 ratio not in the 1.9-2.2 range	Sample was not washed with 1X RNA Wash Buffer	Wash the RNA Spin Cartridge containing bound d-siRNA twice with 1X RNA Wash Buffer (see Steps 9 and 10).

Problem	Reason	Solution
	RNA Spin Cartridge containing bound d-siRNA not centrifuged to remove residual 1X RNA Wash Buffer	Centrifuge RNA Spin Cartridge at 14,000 x g for 1 minute at room temperature to remove residual 1X RNA Wash Buffer and to dry the membrane (see Step 11).

### Transfection and RNAi Analysis

[0380] The table below lists some potential problems and possible solutions that may help you troubleshoot your transfection and knockdown experiment.

Problem	Reason	Solution
Low levels of gene knockdown observed	<p>Low transfection efficiency</p> <ul style="list-style-type: none"> <li>• Antibiotics added to the media during transfection</li> <li>• Cells were confluent at the time of transfection</li> <li>• Not enough d-siRNA transfected</li> <li>• Not enough Lipofectamine™ 2000 used</li> </ul>	<ul style="list-style-type: none"> <li>• Do not add antibiotics to the media during transfection.</li> <li>• Plate cells such that they will be 30-50% confluent at the time of transfection.</li> <li>• Increase the amount of d-siRNA transfected.</li> <li>• Optimize the transfection conditions for your cell line by varying the amount of Lipofectamine™ 2000 used.</li> </ul>

Problem	Reason	Solution
	Didn't wait long enough after transfection before assaying for gene knockdown	<ul style="list-style-type: none"> <li>• Repeat the transfection and wait for a longer period of time after transfection before assaying for gene knockdown.</li> <li>• Perform a time course of expression to determine the point at which the highest degree of gene knockdown occurs.</li> </ul>
	d-siRNA was degraded	<ul style="list-style-type: none"> <li>• Make sure that the d-siRNA is stored in 1X RNA Annealing Buffer.</li> <li>• Aliquot purified d-siRNA and avoid repeated freeze/thaw cycles.</li> </ul>
Cytotoxic effects observed after transfection	Too much Lipofectamine™ 2000 Reagent used	Optimize the transfection conditions for your cell line by varying the amount of Lipofectamine™ 2000 Reagent used.
	Cells transfected with unpurified d-siRNA	<p>Purify d-siRNA using the RNAi Purification reagents supplied with the kit.</p> <p>Transfecting unpurified d-siRNA is not recommended as the contaminating dsRNA will cause host cell shutdown and apoptosis.</p>



Problem	Reason	Solution
No gene knockdown observed	d-siRNA was degraded <ul style="list-style-type: none"> <li>d-siRNA was stored in water</li> <li>d-siRNA was repeatedly frozen and thawed</li> </ul>	<ul style="list-style-type: none"> <li>Make sure that the d-siRNA is stored in 1X RNA Annealing Buffer.</li> <li>Aliquot purified d-siRNA and avoid repeated freeze/thaw cycles.</li> </ul>
	Target region contains no active siRNA	Select a larger target region or a different region.
Non-specific off-target gene knockdown observed	Target sequence contains strong homology to other genes	Select a new target sequence. Limit the size range of the target sequence to 1 kb.

#### Product Qualification

**[0381]** Introduction The components of the BLOCK-iT™ Dicer RNAi Kits are qualified as described below.

#### Functional Qualification

**[0382]** The BLOCK-iT™ Dicer enzyme and RNAi Purification reagents are functionally qualified as follows:

1. The BLOCK-iT™ Dicer enzyme is diluted to 1 U/μl and tested (in triplicate) in a dicing reaction following the procedure above using lacZ dsRNA produced using the BLOCK-iT™ RNAi TOPO® Transcription Kit. Each dicing reaction is assessed by analyzing an aliquot of the reaction on a 20% Novex® TBE gel (Catalog no. EC63152BOX). The 10 bp DNA Ladder (Catalog no. 10821-015) is included as a molecular weight standard. Polyacrylamide gel analysis

should demonstrate a minimal amount of dsRNA remaining in the reaction and minimal to no degradation of siRNA apparent.

2. The dicing reactions are purified using the RNAi purification reagents supplied in the kit and following the procedure above. Purified d-siRNA is quantitated using spectrophotometry. The amount of d-siRNA recovered should be at least 25%.

#### Lipofectamine™ 2000 Reagent

[0383] Lipofectamine™ 2000 is tested for the absence of microbial contamination using blood agar plates, Sabaraud dextrose agar plates, and fluid thioglycolate medium, and functionally by transfection of CHO-K1 cells with a luciferase reporter-containing plasmid.

### BLOCK-iT™ RNAi TOPO® Transcription Kit

#### Introduction

[0384] This quick reference sheet is provided for experienced users of the dsRNA generation procedure. If you are performing the TOPO® Linking, secondary amplification, transcription, purification, or annealing steps for the first time, follow the detailed protocols provided in the manual. We recommend using the pcDNA™1.2/V5-GW/*lacZ* plasmid and the control PCR primers (*lacZ* Forward 2 and *lacZ* Reverse 2 primers) included with the kit to generate dsRNA.

Step	Action
Produce the PCR product	<ol style="list-style-type: none"><li>1. Amplify your sequence of interest using Platinum® <i>Taq</i> DNA polymerase and your own protocol. End the PCR reaction with a final 7 minute extension step.</li><li>2. Use agarose gel electrophoresis to check the integrity and yield of your PCR product.</li></ol>

Step	Action												
Perform the TOPO® Linking reaction	<ol style="list-style-type: none"> <li>Set up the following TOPO® Linking reaction. <table> <tr> <td>Your PCR product (<math>\geq 20</math> ng/<math>\mu</math>l)</td><td>1 <math>\mu</math>l</td></tr> <tr> <td>Salt Solution</td><td>1 <math>\mu</math>l</td></tr> <tr> <td>Sterile water</td><td>3 <math>\mu</math>l</td></tr> <tr> <td><u>BLOCK-iT™ T7-TOPO® Linker</u></td><td><u>1 <math>\mu</math>l</u></td></tr> <tr> <td>Total volume</td><td>6 <math>\mu</math>l</td></tr> </table> </li> <li>Mix reaction gently and incubate for 15 minutes at 37°C.</li> <li>Place the reaction on ice and proceed directly to perform secondary amplification, below.</li> </ol>	Your PCR product ( $\geq 20$ ng/ $\mu$ l)	1 $\mu$ l	Salt Solution	1 $\mu$ l	Sterile water	3 $\mu$ l	<u>BLOCK-iT™ T7-TOPO® Linker</u>	<u>1 <math>\mu</math>l</u>	Total volume	6 $\mu$ l		
Your PCR product ( $\geq 20$ ng/ $\mu$ l)	1 $\mu$ l												
Salt Solution	1 $\mu$ l												
Sterile water	3 $\mu$ l												
<u>BLOCK-iT™ T7-TOPO® Linker</u>	<u>1 <math>\mu</math>l</u>												
Total volume	6 $\mu$ l												
Perform secondary amplification reactions to generate sense and antisense DNA templates	<ol style="list-style-type: none"> <li>Set up 2 PCR reactions - in each reaction, amplify 1 <math>\mu</math>l of the TOPO® Linking reaction using Platinum® <i>Taq</i> DNA polymerase and your own protocol. End the PCR reaction with a final 7 minute extension step. For PCR primers, use the following: <ul style="list-style-type: none"> <li>Sense template: use the BLOCK-iT™ T7 Primer and your gene-specific reverse primer.</li> <li>Antisense template: use the BLOCK-iT™ T7 Primer and your gene-specific forward primer</li> </ul> </li> <li>Use agarose gel electrophoresis to check the integrity and yield of your PCR products.</li> <li>Proceed to perform the RNA transcription reactions, next page.</li> </ol>												
Perform the RNA transcription reaction to generate sense and antisense ssRNA	<ol style="list-style-type: none"> <li>Set up two separate transcription reactions using either the sense or antisense linear DNA template. <table> <tr> <td>RNase-free water</td><td>up to 21 <math>\mu</math>l</td></tr> <tr> <td>75 mM NTPs</td><td>8 <math>\mu</math>l</td></tr> <tr> <td>DNA template (250 ng-1 <math>\mu</math>g)</td><td>1-10 <math>\mu</math>l</td></tr> <tr> <td>10X Transcription buffer</td><td>4 <math>\mu</math>l</td></tr> <tr> <td><u>BLOCK-iT™ T7 Enzyme Mix</u></td><td><u>6 <math>\mu</math>l</u></td></tr> <tr> <td>Total volume</td><td>40 <math>\mu</math>l</td></tr> </table> </li> <li>Incubate the reaction at 37°C for 2 hours.</li> <li>Add 2 <math>\mu</math>l of DNase I to each reaction. Incubate at 37°C for 15 minutes.</li> </ol>	RNase-free water	up to 21 $\mu$ l	75 mM NTPs	8 $\mu$ l	DNA template (250 ng-1 $\mu$ g)	1-10 $\mu$ l	10X Transcription buffer	4 $\mu$ l	<u>BLOCK-iT™ T7 Enzyme Mix</u>	<u>6 <math>\mu</math>l</u>	Total volume	40 $\mu$ l
RNase-free water	up to 21 $\mu$ l												
75 mM NTPs	8 $\mu$ l												
DNA template (250 ng-1 $\mu$ g)	1-10 $\mu$ l												
10X Transcription buffer	4 $\mu$ l												
<u>BLOCK-iT™ T7 Enzyme Mix</u>	<u>6 <math>\mu</math>l</u>												
Total volume	40 $\mu$ l												

Step	Action
Purify the sense and antisense transcripts	<ol style="list-style-type: none"> <li>1. To each RNA transcription reaction, add 160 <math>\mu</math>l of RNA Binding Buffer containing 1% (v/v) <math>\beta</math>-mercaptoethanol followed by 100 <math>\mu</math>l of 100% ethanol. Mix well by pipetting up and down 5 times.</li> <li>2. Apply the sample to the RNA Spin Cartridge, and centrifuge at 14,000 x g for 15 seconds at room temperature. Discard the flow-through.</li> <li>3. Add 500 <math>\mu</math>l of 1X RNA Wash Buffer to the RNA Spin Cartridge, and centrifuge at 14,000 x g for 15 seconds at room temperature. Discard the flow-through.</li> <li>4. Repeat Step 3.</li> <li>5. Centrifuge the RNA Spin Cartridge at 14,000 x g for 1 minute at room temperature.</li> <li>6. Remove the RNA Spin Cartridge from the Wash Tube, and place it in an RNA Recovery Tube. Add 40 <math>\mu</math>l of RNase-free water to the RNA Spin Cartridge. Let stand at room temperature for 1 minute, then centrifuge the RNA Spin Cartridge at 14,000 x g for 2 minutes at room temperature to elute the ssRNA.</li> <li>7. Add 40 <math>\mu</math>l of RNase-Free Water to the RNA Spin Cartridge and repeat Step 7, eluting the ssRNA into the same RNA Recovery Tube. Add 1.4 <math>\mu</math>l of 50X RNA Annealing Buffer to the eluted ssRNA.</li> <li>8. Quantitate the yield of ssRNA by spectrophotometry.</li> </ol>
Anneal the sense and antisense transcripts to produce dsRNA	<ol style="list-style-type: none"> <li>1. In a microcentrifuge tube, mix equal amounts of purified sense and antisense ssRNA.</li> <li>2. Heat 250 ml of water to boiling in a 500 ml glass beaker, remove from the heat, and set the beaker on the laboratory bench.</li> <li>3. Place the tube containing the ssRNA mixture (in a tube float) in the glass beaker and allow the water to cool to room temperature for 1-1.5 hours.</li> <li>4. Aliquot and store the dsRNA at -80°C.</li> </ol>

#### Kit Contents and Storage

#### Types of Kits

[0385] This manual is supplied with the products listed below.

- [0386] The BLOCK-iT™ Complete Dicer RNAi Kit is also supplied with the BLOCK-iT™ Dicer RNAi Transfection Kit and the BLOCK-iT™ Dicer RNAi Kits manual.

Product	Catalog no.
BLOCK-iT™ RNAi TOPO® Transcription Kit	K3500-01
BLOCK-iT™ Complete Dicer RNAi Kit	K3650-01

#### Kit Components

- [0387] The BLOCK-iT™ RNAi Kits include the following components. For a detailed description of the contents of the BLOCK-iT™ RNAi TOPO® Transcription Kit.

- [0388] The BLOCK-iT™ Complete Dicer RNAi Kit also includes the BLOCK-iT™ Dicer RNAi Transfection Kit. For a detailed description of the reagents supplied in the BLOCK-iT™ Dicer RNAi Transfection Kit, refer to the BLOCK-iT™ Dicer RNAi Kits manual.

Component	Catalog no.	
	<u>K3500-01</u>	<u>K3650-01</u>
BLOCK-iT™ RNAi TOPO® Transcription Kit	√	√
BLOCK-iT™ Dicer RNAi Transfection Kit		√

#### Shipping/Storage

- [0389] The BLOCK-iT™ RNAi TOPO® Transcription Kit is shipped as described below. Upon receipt, store each item as detailed below.

Box	Component	Shipping	Storage
1	BLOCK-iT™ TOPO® Linker Kit	Dry ice	-20°C
2	BLOCK-iT™ RNAi Transcription Kit	Dry ice	-20°C
3	BLOCK-iT™ RNAi Purification Kit	Room temperature	Room temperature

# BLOCK-iT™ TOPO® Linker Kit Reagents

[0390] The following reagents are supplied with the BLOCK-iT™ TOPO® Linker Kit (Box 1). Note that the user must supply *Taq* polymerase. Store the reagents at -20°C.

Reagent	Composition	Amount
BLOCK-iT™ T7-TOPO® Linker	0.1-1 ng/μl double-stranded DNA in: 50 mM Tris-HCl, pH 7.3 100 mM NaCl 0.2 mM EDTA 0.9 mM DTT 45 μg/ml BSA 0.05% (v/v) Triton X-100 40% (v/v) glycerol	5 μl
10X PCR Buffer	100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM MgCl <sub>2</sub> 0.01% gelatin	75 μl
40 mM dNTPs	10 mM dATP 10 mM dTTP 10 mM dGTP 10 mM dCTP neutralized at pH 8.0 in water	15 μl
Salt Solution	1.2 M NaCl 0.06 M MgCl <sub>2</sub>	10 μl
Sterile Water	--	750 μl
BLOCK-iT™ T7 Primer	75 ng/μl in TE Buffer, pH 8.0	10 μl
LacZ Forward 2 Primer	65 ng/μl in TE Buffer, pH 8.0	10 μl
LacZ Reverse 2 Primer	65 ng/μl in TE Buffer, pH 8.0	10 μl
pcDNA™1.2/V5-GW/ <i>lacZ</i> control plasmid	Lyophilized in TE Buffer, pH 8.0	10 μg

## Primer Sequences

[0391] The table below provides the sequence and the amount supplied of the primers included in the kit.

Primer	Sequence	Amount
BLOCK-iT™ T7	5'-GATGACTCGTAATACGACTCACTA-3' (SEQ ID NO. 1)	103 pmoles
LacZ Forward 2	5'-ACCAGAAGCGGTGCCGAAA-3' (SEQ ID NO. 2)	105 pmoles
LacZ Reverse 2	5'-CCACAGCGGATGGTTCGGAT-3' (SEQ ID NO. 3)	106 pmoles

## BLOCK-iT™ RNAi Transcription Kit Reagents

[0392] The following reagents are included with the BLOCK-iT™ RNAi Transcription Kit. Store reagents at -20°C.

Reagent	Composition	Amount
BLOCK-iT™ T7 Enzyme Mix		60 µl
10X Transcription Buffer		40 µl
75 mM NTPs	18.75 mM ATP 18.75 mM UTP 18.75 mM CTP 18.75 mM GTP neutralized at pH 8.0 in water	80 µl
RNase-Free Water	—	800 µl
DNase I	1 U/µl in 20 mM sodium acetate, pH 6.5 5 mM CaCl <sub>2</sub> 0.1 mM PMSF 50% (v/v) glycerol	20 µl

## BLOCK-iT™ RNAi Purification Kit

[0393] The following reagents are included with the BLOCK-iT™ RNAi Purification Kit. Store reagents at room temperature. Use caution when handling the RNA Binding Buffer.

**[0394]** Catalog no. K3650-01 includes two boxes of BLOCK-iT™ RNAi Purification reagents. One box is supplied with the BLOCK-iT™ RNAi TOPO® Transcription Kit for purification of the single-stranded RNA (ssRNA). The second box is supplied with the BLOCK-iT™ Dicer RNAi Transfection Kit for purification of diced siRNA (d-siRNA).

Reagent	Composition	Amount
RNA Binding Buffer		1.8 ml
5X RNA Wash Buffer		2.5 ml
RNase-Free Water	--	800 µl
RNA Spin Cartridges	--	10
RNA Recovery Tubes	--	10
siRNA Collection Tubes*	--	5
50X RNA Annealing Buffer	500 mM Tris-HCl, pH 8.0 1 M NaCl 50 mM EDTA, pH 8.0	50 µl

siRNA Collection Tubes are not required for the purification of the ssRNA, and are used for purification of d-siRNA only.

**[0395]** The RNA Binding Buffer supplied in the BLOCK-iT™ RNAi Purification Kit contains guanidine isothiocyanate. This chemical is harmful if it comes in contact with the skin or is inhaled or swallowed. Always wear a laboratory coat, disposable gloves, and goggles when handling solutions containing this chemical.

**[0396]** Do not add bleach or acidic solutions directly to solutions containing guanidine isothiocyanate or sample preparation waste. Guanidine isothiocyanate forms reactive compounds and toxic gases when mixed with bleach or acids.



## Accessory Products

[0397] The table below provides ordering information for products available from Invitrogen that are suitable for use with the BLOCK-iT™ RNAi TOPO® Transcription Kit.

Item	Amount	Catalog no.
BLOCK-iT™ Dicer RNAi Transfection Kit	5 genes x 150 transfections each*	K3600-01
<i>Taq</i> DNA Polymerase, Native	100 units	18038-018
	500 units	18038-042
<i>Taq</i> DNA Polymerase, Recombinant	100 units	10342-053
	500 units	10342-020
Platinum® <i>Taq</i> DNA Polymerase	100 reactions	10966-018
	250 reactions	10966-026
	500 reactions	10966-034
6% Novex® TBE Gel	1 box	EC6265BOX
0.16-1.77 kb RNA Ladder	75 µg	15623-010

\* Based on transfection in 24-well plates.

## Introduction

[0398] The BLOCK-iT™ RNAi TOPO® Transcription Kit facilitates rapid generation of T7 promoter-based DNA templates. Using the DNA templates and reagents supplied with the kit, RNA transcripts are produced, purified, and annealed to generate double-stranded RNA (dsRNA). The resulting dsRNA may be used directly for RNA interference (RNAi) analysis in invertebrate systems and other systems lacking the interferon response or as a substrate to produce short interfering RNA (siRNA) for RNAi analysis in mammalian cells.

## Advantages of the BLOCK-iT™ RNAi TOPO® Transcription Kit

[0399] Use of the BLOCK-iT™ RNAi TOPO® Transcription Kit to facilitate production of dsRNA provides the following advantages:

The BLOCK-iT™ T7-TOPO® Linker provides a method to quickly and easily add a T7 promoter to any existing Taq-amplified PCR product without the need for new primers or subcloning.

Use of the TOPO® Linking Technology and secondary amplification enables simultaneous production of linear DNA templates that may be used directly for *in vitro* transcription to generate sense and antisense transcripts. Creation of a T7 expression plasmid, bacterial transformation, and plasmid purification are not required.

Separate transcription reactions using sense and antisense templates allow precise quantitation of ssRNA concentration prior to annealing.

Provides optimized purification reagents to obtain highly pure sense and antisense transcripts that can be annealed to generate an optimal yield of dsRNA. Double-stranded RNA can be used directly for RNAi analysis in invertebrate systems or as a substrate for the Dicer enzyme to generate siRNA.

[0400] This manual provides instructions and guidelines to:

1. Amplify your sequence of interest and use TOPO® Linking to join the primary PCR product to the BLOCK-iT™ T7-TOPO® Linker.
2. Use the appropriate primers to amplify the TOPO® Linked PCR product to generate linear sense and antisense DNA templates.
3. Use the linear sense and antisense DNA templates in transcription reactions to generate sense and antisense single-stranded RNA (ssRNA) transcripts of the sequence of interest.
4. Purify the sense and antisense ssRNA transcripts and anneal them to generate dsRNA. The resulting dsRNA may then be used in the application of choice (e.g. RNAi analysis in invertebrate organisms or as a substrate for “dicing” to produce d-siRNA for RNAi analysis in mammalian cells).

[0401] For details and instructions to generate d-siRNA using Dicer, refer to the BLOCK-iT™ Dicer RNAi Kits manual. This manual is supplied with the BLOCK-iT™ Dicer RNAi Transfection and Complete Dicer RNAi Kits.

[0402] The BLOCK-iT™ RNAi TOPO® Transcription Kit is designed to help you generate dsRNA for direct use in RNAi analysis in invertebrate systems or as a substrate in a dicing reaction to produce d-siRNA for RNAi analysis in mammalian cells. Although the kit has been designed to help you generate dsRNA representing a particular target sequence in the simplest, most direct fashion, use of the resulting dsRNA for RNAi analysis assumes that users are familiar with the mechanism of gene silencing and the techniques that exist to introduce dsRNA into the organism or cell type of choice. We highly recommend that users possess a working knowledge of the RNAi pathway and the methodologies required to perform RNAi analysis in the organism or cell type of choice.

[0403] For more information about these topics, refer to published reviews (Bosher and Labouesse, 2000; Hannon, 2002; Plasterk and Ketting, 2000; Zamore, 2001). A variety of BLOCK-iT™ RNAi products are available from Invitrogen to facilitate your RNAi analysis.

#### Description of the System

[0404] The BLOCK-iT™ RNAi TOPO® Transcription Kit facilitates generation of T7 promoter-based DNA templates for in vitro transcription and production of dsRNA, and consists of three major components:

1. The BLOCK-iT™ T7-TOPO® Linker for quick and easy creation of T7 promoter-based DNA templates for in vitro transcription. Using TOPO® Linking Technology, the BLOCK-iT™ T7-TOPO® Linker may be linked to any Taq-amplified PCR product. The linked PCR product is then amplified to generate a linear DNA template.
2. BLOCK-iT™ RNAi Transcription reagents for generation of sense and antisense ssRNA transcripts from your T7-based, linear DNA template. The reagents include an optimized T7 Enzyme Mix for highly efficient production of ssRNA.

3. The BLOCK-iT™ RNAi Purification reagents for silica-based column purification of sense and antisense ssRNA transcripts, and an RNA Annealing Buffer to stabilize dsRNA duplexes for long-term storage.

[0405] The BLOCK-iT™ RNAi TOPO® Transcription Kit also includes a control expression plasmid containing the lacZ gene and PCR primers that may be used as controls to generate dsRNA. Once generated, the lacZ dsRNA may be used for the following types of RNAi analysis:

#### Invertebrate Systems

[0406] As a negative control for non-specific gene knockdown in any invertebrate system. The lacZ dsRNA is not suitable for use as a positive control to knock down  $\beta$ -galactosidase expression from the control pcDNA™1.2/V5-GW/lacZ plasmid in any invertebrate system. This is because expression of the lacZ gene from the control plasmid is controlled by the human cytomegalovirus (CMV) promoter, and this promoter is not active in most invertebrate systems.

#### Mammalian Systems

[0407] As a negative control for non-specific gene knockdown or as a positive control for knockdown of  $\beta$ -galactosidase expression from the pcDNA™1.2/V5-GW/lacZ reporter plasmid. Note that to perform RNAi analysis in mammalian cells, the lacZ dsRNA should first be “diced” to generate d-siRNA. For details, refer to the BLOCK-iT™ Dicer RNAi Kits manual.

#### Generating dsRNA Using the BLOCK-iT™ RNAi TOPO® Transcription Kit

[0408] You will perform the following steps to generate dsRNA using the BLOCK-iT™ RNAi TOPO® Transcription Kit. For a diagram, see Figure 12 illustrating the major steps necessary to generate dsRNA using the BLOCK-iT™ RNAi TOPO® Transcription System..

1. Amplify your sequence of interest using Taq polymerase.
2. Perform a TOPO® Linking reaction to link your PCR product to the BLOCK-iT™ T7-TOPO® Linker containing the T7 promoter.
3. Using a combination of the BLOCK-iT™ T7 Primer (supplied with the kit) and your gene-specific forward or reverse primer, amplify the TOPO® Linked PCR product with Taq polymerase to produce linear sense and antisense DNA templates.
4. Use the sense and antisense DNA templates and the reagents supplied in the kit in an in vitro transcription reaction to produce sense and antisense RNA transcripts, respectively.
5. Purify the sense and antisense RNA transcripts using the RNAi Purification reagents supplied in the kit.
6. Quantitate the yield of purified sense and antisense ssRNA transcripts, and anneal equal amounts of each single-stranded transcript to form dsRNA.

#### How TOPO® Linking Works

#### How Topoisomerase I Works

[0409] Topoisomerase I from Vaccinia virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5'-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Linking exploits this reaction to efficiently join PCR products to the BLOCK-iT™ T7-TOPO® Linker.

### TOPO® Linking

- [0410] The BLOCK-iT™ T7-TOPO® Linker is supplied linearized with:  
A single 3' thymidine (T) overhang for TA Cloning®  
Topoisomerase I covalently bound to the linker (this is referred to as "activated linker")
- [0411] Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linear BLOCK-iT™ T7-TOPO® linker supplied in this kit has a single, overhanging 3' deoxythymidine (T) residue. This allows PCR products to ligate efficiently with the linker.
- [0412] TOPO® Linking as shown in Figure 13 exploits the ligation activity of topoisomerase I by providing an "activated" linearized TA linker (Shuman, 1994). Ligation of the linker with a PCR product containing 3' A-overhangs is very efficient and occurs spontaneously with maximum efficiency at 37°C within 15 minutes.

### The RNAi Pathway

- [0413] RNAi describes the phenomenon by which dsRNA induces potent and specific inhibition of eukaryotic gene expression via the degradation of complementary messenger RNA (mRNA), and is functionally similar to the processes of post-transcriptional gene silencing (PTGS) or cosuppression in plants (Cogoni *et al.*, 1994; Napoli *et al.*, 1990; Smith *et al.*, 1990; van der Krol *et al.*, 1990) and quelling in fungi (Cogoni and Macino, 1999; Cogoni and Macino, 1997; Romano and Macino, 1992). In plants, the PTGS response is thought to occur as a natural defense against viral infection or transposon insertion (Anandalakshmi *et al.*, 1998; Jones *et al.*, 1998; Li and Ding, 2001; Voinnet *et al.*, 1999).
- [0414] In eukaryotic organisms, dsRNA produced *in vivo* or introduced by pathogens is processed into 21-23 nucleotide double-stranded short interfering RNA duplexes (siRNA) by an enzyme called Dicer, a member of the RNase

III family of double-stranded RNA-specific endonucleases (Bernstein *et al.*, 2001; Ketting *et al.*, 2001). The siRNA then incorporate into the RNA-induced silencing complex (RISC), a second enzyme complex that serves to target cellular transcripts complementary to the siRNA for specific cleavage and degradation (Hammond *et al.*, 2000; Nykanen *et al.*, 2001).

- [0415] For more information about the RNAi pathway and the mechanism of gene silencing, refer to reviews (Bosher and Labouesse, 2000; Hannon, 2002; Plasterk and Ketting, 2000; Zamore, 2001).

#### Using the Kit for RNAi Analysis

- [0416] The BLOCK-iT™ RNAi TOPO® Transcription Kit facilitates *in vitro* production of dsRNA that is targeted to a particular gene of interest. The long dsRNA is introduced into the appropriate organism or cells, where the endogenous Dicer enzyme processes the dsRNA into siRNA. The resulting siRNA can then inhibit expression of the target gene. For a diagram of the process, see Figure 14.

#### Use of dsRNA for RNAi Analysis

- [0417] Long dsRNA duplexes can be used directly for RNAi analysis in organisms and systems lacking the interferon response, including insects (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999), insect cell lines (Caplen *et al.*, 2000), *C. elegans* (Fire *et al.*, 1998), trypanosomes (Ngo *et al.*, 1998), some mammalian embryonic cell lines (Billy *et al.*, 2001; Yang *et al.*, 2001), and mouse oocytes and preimplantation embryos (Svoboda *et al.*, 2000; Wianny and Zernicka-Goetz, 2000).
- [0418] Long dsRNA duplexes cannot be used directly for RNAi analysis in most somatic mammalian cell lines. This is because introduction of dsRNA into these cell lines induces a non-specific, interferon-mediated response resulting in shutdown of translation and initiation of cellular apoptosis (Kaufman, 1999). To perform RNAi analysis in mammalian cell lines, long dsRNA should first be cleaved into 21-23 nucleotide siRNA duplexes. This

cleavage process may be performed in vitro using recombinant Dicer enzyme such as is provided in the BLOCK-iT™ Dicer RNAi Transfection Kit or the BLOCK-iT™ Complete Dicer RNAi Kit. For more information, refer to the BLOCK-iT™ Dicer RNAi Kits manual.

#### Experimental Outline

[0419] The table below describes the major desired steps to generate a dsRNA using the BLOCK-iT™ RNAi TOPO® Transcription Kit.

Step	Action
1	Produce your PCR product using <i>Taq</i> polymerase or Platinum® <i>Taq</i> DNA polymerase.
2	Verify the integrity and concentration of your PCR product.
3	Perform the TOPO® Linking reaction to link your PCR product to the BLOCK-iT™ T7-TOPO® Linker.
4	Amplify the TOPO® Linked PCR product using the appropriate primers to produce sense and antisense linear DNA templates.
5	Use each linear DNA template in an RNA transcription reaction to produce sense and antisense RNA transcripts.
6	Purify sense and antisense RNA transcripts.
7	Quantitate the yield of each purified ssRNA obtained, and anneal equal amounts of sense and antisense ssRNA to generate dsRNA.

#### Methods

##### Designing PCR Primers

[0420] To use the BLOCK-iT™ RNAi TOPO® Transcription Kit, you will first need to design PCR primers to amplify your sequence of interest. Guidelines to choose the target sequence and to design PCR primers are provided below.



### Choosing the Target Sequence

[0421] When performing RNAi analysis, your choice of target sequence can significantly affect the degree of gene knockdown observed. In addition, the size of the target sequence and the resulting dsRNA can affect the transcription efficiency and thus the yield of dsRNA produced. Consider the following factors when choosing your target sequence.

1. Select a target sequence that covers a reasonable portion of the gene of interest and that does not contain regions of strong homology with other genes.
2. Limit the size of the target sequence. Although smaller or larger target sequences are possible, we recommend limiting the initial target sequence to a size range of 500 bp to 1 kb for the following reasons.
  - (a) This balances the risk of including regions of strong homology between the target gene and other genes that could result in non-specific off-target effects during RNAi analysis with the benefits of using a more complex pool of siRNA.
  - (b) When producing sense and antisense transcripts of the target template, the highest transcription efficiencies are obtained with transcripts in the 500 bp to 1 kb size range. Target templates outside this size range transcribe less efficiently, resulting in lower yields of dsRNA.
  - (c) If you plan to “dice” the dsRNA to produce d-siRNA for use in mammalian RNAi analysis, note that dsRNA that are under 1 kb in size are efficiently diced. Larger dsRNA can be used but yields may decline as the size increases.

[0422] The BLOCK-iT™ Complete Dicer RNAi Kit has been used successfully to knock down gene activity with dsRNA substrates ranging from 150 bp to 1.3 kb in size.

### Factors to Consider When Designing PCR Primers

[0423] Once you have selected an appropriate target sequence, you will need to design gene-specific primers to amplify your target sequence of interest. Consider the following factors when designing gene-specific primers.

1. Make sure that your primers do not contain sequence that is homologous to other genes.
2. Once you have linked your primary PCR product to the BLOCK-iT™ T7-TOPO® Linker, you will amplify the resulting linked product using the BLOCK-iT™ T7 Primer and either your gene-specific forward primer or gene-specific reverse primer. When designing your gene-specific PCR primers, make sure that the  $T_m$  of each primer is compatible with the  $T_m$  of the BLOCK-iT™ T7 primer (i.e.  $T_m = 62^\circ\text{C}$ ).

[0424] Figure 15 can be used to design appropriate PCR primers to join your sequence of interest with the BLOCK-iT™ T7-TOPO® Linker. The BLOCK-iT™ T7-TOPO® Linker is supplied as a double-stranded DNA fragment adapted with topoisomerase I.

[0425] Features of the BLOCK-iT™ T7-TOPO® Linker:

The sequence of the T7 promoter is indicated in bold.

The transcription start site is indicated by +1.

[0426] To obtain consistent and efficient results in the TOPO® Linking reaction, we recommend using HPLC-purified oligonucleotides to produce your PCR products. Using a mixture of full-length and non full-length primers to produce your PCR products can reduce the efficiency of TOPO® Linking and result in poor yield of the linear DNA templates after secondary amplification.

[0427] Do not add 5' phosphates to your primers for PCR. This will prevent TOPO® Linking.

### Amplifying Your Sequence of Interest

- [0428] Once you have decided on a PCR strategy and have synthesized the primers, you are ready to produce your PCR product.

### Choosing a Thermostable DNA Polymerase

- [0429] To amplify your sequence of interest, use a thermostable DNA polymerase that generates PCR products with 3' A-overhangs. We recommend using Platinum® Taq polymerase available from Invitrogen. Taq polymerase is also suitable.
- [0430] You may use Taq polymerase and proofreading polymerase mixtures to generate PCR products, however, a certain proportion of your PCR products will be blunt-ended. You can add 3' A-overhangs to your PCR products using the method below.

### Control Plasmid

- [0431] We recommend amplifying the control template included with the kit in parallel with your sample. Use the LacZ Forward 2 and the LacZ Reverse 2 primers included with the kit to amplify the pcDNA™1.2/V5-GW/*lacZ* plasmid. The resulting control PCR product (representing a 1 kb fragment of the *lacZ* gene) may then be used as a positive control for subsequent procedures including TOPO® Linking, transcription, and production of dsRNA. For a map of pcDNA™1.2/V5-GW/*lacZ*, refer to Figure 17.
- [0432] To use the pcDNA™1.2/V5-GW/*lacZ* plasmid as a template for amplification, resuspend the plasmid in 10 µl of sterile water to obtain a final concentration of 1 µg/µl. Dilute as appropriate and use 1-10 ng of plasmid DNA in the PCR reaction.

### Materials Needed

- [0433] You should have the following materials on hand before beginning:  
Thermocycler

Thermostable DNA polymerase (e.g. Platinum® Taq DNA Polymerase)

DNA template

Gene-specific forward and reverse PCR primers (10  $\mu$ M each)

10X PCR Buffer (supplied with the kit, Box 1)

40 mM dNTPs (supplied with the kit, Box 1)

Sterile water (supplied with the kit, Box 1)

#### Setting Up the PCR Reaction

[0434] Use the procedure below to amplify your sequence of interest using Platinum® Taq DNA polymerase. Use less DNA if you are using plasmid DNA as a template (1-10 ng) and more DNA if you are using genomic DNA as a template (10-100 ng).

[0435] If you are using a different thermostable DNA polymerase, reaction conditions may vary.

1. Set up the following 50  $\mu$ l PCR reaction.

DNA Template	1-100 ng
10X PCR Buffer	5 $\mu$ l
40 mM dNTPs	1 $\mu$ l
PCR Primers (10 $\mu$ M each)	1 $\mu$ l each
Sterile water	add to a final volume of 49.5 $\mu$ l
<u>Platinum® Taq polymerase (5 U/<math>\mu</math>l)</u>	<u>0.5 <math>\mu</math>l</u>
Total volume	50 $\mu$ l

2. Use the cycling parameters suitable for your primers and template. Be sure to include a 7 minute extension at 72°C after the last cycle to ensure that all PCR products are full-length and 3' adenylated.
3. After cycling, place the tube on ice. Proceed to Checking the PCR Product, below.

### Checking the PCR Product

[0436] Analyze 1-5  $\mu$ l of the PCR reaction using agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following:

1. A single discrete band of the expected size corresponding to your sequence of interest. If you do not obtain a single, discrete band from your PCR, follow the manufacturer's recommendations or use the PCR Optimizer™ Kit (Catalog no. K1220-01) from Invitrogen to optimize your PCR conditions using your DNA polymerase. Other tips may be found below or in published reference sources (Innis *et al.*, 1990). Alternatively, you may gel-purify your fragment before proceeding to TOPO® Linking.
2. Estimate the concentration of your PCR product. For optimal TOPO® Linking, the concentration of your PCR should be  $\geq 20$  ng/ $\mu$ l. If your PCR product is too dilute, see Concentrating Dilute PCR Products, below.

[0437] Once you have verified that your PCR product is of the appropriate quality and concentration, proceed to Performing the TOPO® Linking Reaction.

[0438] For optimal results, use fresh PCR product in the TOPO® Linking reaction.

[0439] You may store the PCR product at -20°C for up to 1 week.

### Concentrating Dilute PCR Products

[0440] If you obtain a single band from PCR, but your PCR product is too dilute, you may purify and concentrate the PCR product before proceeding to the TOPO® Linking reaction. A procedure to purify and concentrate PCR products is provided below.

## Performing the TOPO® Linking Reaction

### Introduction

**[0441]** Once you have produced your PCR product, you will use TOPO® Linking to join the PCR product to the BLOCK-iT™ T7-TOPO® Linker. Before performing the TOPO® Linking reaction, you should have everything you need set up and ready to use to ensure that you obtain the best results. If you have produced the control PCR product and this is the first time you have performed TOPO® Linking, we recommend performing the control TOPO® Linking reaction below in parallel with your samples.

### Materials Needed

**[0442]** Have the following reagents on hand before beginning:

- Your primary PCR product ( $\geq 20$  ng/ $\mu$ l)
- BLOCK-iT™ T7-TOPO® Linker (supplied with the kit, Box 1; keep at -20°C until use)
- Salt Solution (supplied with the kit; Box 1)
- Sterile Water (supplied with the kit; Box 1)
- 37°C water bath

### TOPO® Linking Procedure

**[0443]** Follow the procedure below to perform the TOPO® Linking reaction.

1. Set up a 6  $\mu$ l TOPO® Linking reaction using the following reagents in the order given.

Your PCR product ( $\geq 20$ ng/ $\mu$ l)	1 $\mu$ l
Salt Solution	1 $\mu$ l
Sterile water	3 $\mu$ l
<u>BLOCK-iT™ T7-TOPO® Linker</u>	<u>1 <math>\mu</math>l</u>
Total volume	6 $\mu$ l
2. Mix reaction gently and incubate for 15 minutes at 37°C.

Do not incubate the reaction for longer than 15 minutes as this may negatively affect TOPO® Linking.

3. Place the reaction on ice and proceed directly to Performing Secondary Amplification.

You may store the TOPO® Linking reaction at -20°C overnight, if desired.

### Performing Secondary Amplification Reactions

#### Introduction

- [0444] Once you have performed the TOPO® Linking reaction, you will use this reaction mixture in two PCR reactions with the appropriate PCR primers to produce sense and antisense linear DNA templates. Guidelines to perform secondary amplification are provided in this section.

#### Thermostable DNA Polymerase

- [0445] You may use any thermostable DNA polymerase to produce sense and antisense linear DNA templates. We generally use the same thermostable DNA polymerase to perform secondary amplification as we use to generate the primary PCR product (i.e. Platinum® Taq DNA Polymerase).

#### PCR Primers

- [0446] To produce sense and antisense linear DNA templates, you will perform two amplification reactions using the TOPO® Linking reaction and the appropriate primers (see table below). For gene-specific PCR primers, use the primers that you used to produce your primary PCR product. The BLOCK-iT™ T7 Primer is supplied with the kit.

Sense Template	Antisense Template
BLOCK-iT™ T7 Primer	BLOCK-iT™ T7 Primer
Gene-specific reverse primer	Gene-specific forward primer

## General Guidelines

[0447] When amplifying the TOPO® Linked PCR product, we recommend the following:

Perform the PCR reaction in a total volume of 50 µl.

Use 1 µl of the TOPO® Linking reaction as the DNA template.

If you use the same thermostable DNA polymerase to perform secondary amplification as was used to generate the primary PCR product, you may generally use similar cycling conditions. However, because you are using different PCR primers, you may need to adjust the cycling conditions.

## Materials Needed

[0448] You should have the following materials on hand before beginning:

Thermocycler

Thermostable DNA polymerase (e.g. Platinum® Taq DNA Polymerase)

TOPO® Linking reaction (from Step 3)

Gene-specific forward and reverse primers (10 µM each)

BLOCK-iT™ T7 Primer (supplied with the kit, Box 1)

10X PCR Buffer (supplied with the kit, Box 1)

40 mM dNTPs (supplied with the kit, Box 1)

Sterile water (supplied with the kit, Box 1)

## Setting Up the Secondary PCR Reactions

[0449] Use the procedure below to amplify the TOPO® Linked PCR product using Platinum® Taq DNA polymerase. If you are using a different thermostable DNA polymerase, reaction conditions may vary.

1. Set up the following 50 µl PCR reactions:

Reagent	Sense Template	Antisense Template
10X PCR Buffer	5 µl	5 µl
40 mM dNTPs	1 µl	1 µl



BLOCK-iT™ T7 Primer (75 ng/μl)	1 μl	1 μl
Gene-specific forward primer (10 μM)	—	1 μl
Gene-specific reverse primer (10 μM)	1 μl	—
Sterile water	40.5 μl	40.5 μl
TOPO® Linking reaction	1 μl	1 μl
Platinum® <i>Taq</i> Polymerase (5 U/μl)	0.5 μl	0.5 μl
Total volume	50 μl	50 μl

2. Use the cycling parameters suitable for your primers and template. Be sure to include a 7 minute extension at 72°C after the last cycle to ensure that all PCR products are full-length.
3. After cycling, place the tube on ice. Proceed to Checking the PCR Products, below.

#### Checking the PCR Products

**[0450]** Analyze 1-5 μl of each PCR reaction using agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following:

A single discrete band of the expected size corresponding to your linked linear DNA template.

You may see some minor background bands. These are generally due to smaller PCR products that were in the primary PCR reaction and should not affect the efficiency of the transcription reaction.

Estimate the concentration of each PCR product. For optimal transcription efficiency, the concentration of each PCR product should be  $\geq 25$  ng/μl. If your PCR product(s) is too dilute, you may increase the number of cycles of the amplification reaction or use the procedure provided below to purify and concentrate your PCR product.

**[0451]** Once you have verified that your PCR products are of the appropriate quality and concentration, proceed to Performing the RNA Transcription Reaction.

### Storing the PCR Products

- [0452] For optimal results, use fresh PCR products in the RNA transcription reaction. You may store the PCR products at -20°C for up to 1 month, if desired.

### Performing the RNA Transcription Reactions

- [0453] Once you have produced the sense and antisense DNA templates of your target sequence, you will perform two transcription reactions using the reagents supplied in the RNA Transcription Kit (Box 2) to generate sense and antisense transcripts.

### Amount of DNA Template to Use

- [0454] For each RNA transcription reaction, you will need 250 ng to 1 µg of your DNA template. For best results, make sure that the concentration of your sense and antisense DNA templates is  $\geq 25$  ng/µl.

### Positive Control

- [0455] If you have performed the control reactions described, we recommend using the resulting sense and antisense lacZ templates as controls in the RNA transcription, purification, and annealing procedures. Once you have produced control lacZ dsRNA, you may:

Use this dsRNA as a negative control for non-specific, off-target effects in your RNAi studies.

Include the lacZ dsRNA in a dicing reaction (refer to the BLOCK-iT™ Dicer RNAi Kits manual for instructions), then use the resulting lacZ d-siRNA as a positive control for RNAi in mammalian cells. Co-transfect the lacZ d-siRNA and the pcDNA™1.2/V5-GW/lacZ plasmid into mammalian cells and assay for knockdown of  $\beta$ -galactosidase expression.

- [0456] When performing the RNA transcription reaction and all subsequent procedures, take precautions to avoid RNase contamination.

Use RNase-free, sterile pipette tips and supplies for all manipulations.

Use DEPC-treated solutions as necessary.

Wear gloves when handling reagents and solutions and when setting up the transcription reaction.

#### Materials Needed

- [0457] You should have the following materials on hand before beginning:
- Sense and antisense DNA templates (from the Secondary Amplification reactions, Step 3;  $\geq 25$  ng/ $\mu$ l each)
  - RNase-Free Water (supplied with the kit, Box 2)
  - 75 mM NTPs (supplied with the kit, Box 2)
  - 10X Transcription Buffer (supplied with the kit, Box 2; keep on ice until use)
  - BLOCK-iT™ T7 Enzyme Mix (supplied with the kit, Box 2; keep at -20°C until use)
  - DNase I (supplied with the kit, Box 2)
  - RNase-free supplies (e.g. microcentrifuge tubes and pipette tips)
  - 37°C water bath

#### Guidelines to Set Up the Transcription Reactions

- [0458] Follow the guidelines below when setting up the transcription reactions.
- Set up the transcription reaction at room temperature. Do not set up the reaction on ice as components in the transcription buffer may precipitate the DNA template.
- Keep the 10X Transcription Buffer on ice; do not thaw until immediately before use.
- Upon thawing the 10X Transcription Buffer, you may notice some precipitate in the bottom of the tube. Warm the buffer to 37°C and vortex briefly to allow the precipitate to go back into solution.
- When setting up the transcription reaction, add the components to the microcentrifuge tube exactly in the order stated. Add the 10X Transcription

Buffer to the mixture directly before adding the BLOCK-iT™ T7 Enzyme Mix, and mix immediately to avoid precipitation of the template. After use, return the 10X Transcription Buffer and the BLOCK-iT™ T7 Enzyme Mix to -20°C.

#### RNA Transcription Procedure

[0459] Use the procedure below to synthesize transcripts from your DNA template. Remember that for each gene, you will generate sense and antisense transcripts using the sense and antisense DNA templates, respectively. Be sure to use RNase-free supplies and wear gloves to prevent RNase contamination.

[0460] If you wish to include a negative control, set up the transcription reaction as described below, except omit the DNA template.

1. For each sample, add the following components exactly in the order stated to a 0.5 ml sterile, microcentrifuge tube at room temperature and mix. The amount of RNase-free water added will depend on the concentration of your DNA template.

Reagents	Amount
RNase-Free Water	up to 21 µl
75 mM NTPs	8 µl
DNA template (250 ng-1 µg)	1-10 µl
10X Transcription Buffer	4 µl
BLOCK-iT™ T7 Enzyme Mix	6 µl
Total volume	40 µl

2. Incubate the reaction at 37°C for 2 hours.

The length of the RNA transcription reaction can be extended up to 6 hours. Most of the transcripts are produced within the first 2 hours, but yields can be increased with longer incubation.

3. Add 2 µl of DNase I to each reaction. Incubate for 15 minutes at 37°C.
4. Proceed to Purifying RNA Transcripts.

You may store the RNA transcription reactions at -20°C overnight before purification, if desired.

## Purifying RNA Transcripts

- [0461] This section provides guidelines and instructions to purify the single-stranded RNA transcripts (ssRNA) produced in the RNA transcription reaction. Use the BLOCK-iT™ RNA Purification reagents (Box 3) supplied with the kit. Remember that for each gene, you will perform 2 purification reactions to purify sense and antisense RNA transcripts.

## Experimental Outline

- [0462] To purify RNA transcripts, you will:
1. Add RNA Binding Buffer and ethanol to the transcription reaction to denature the proteins and to enable the ssRNA to bind to the column.
  2. Add the sample to an RNA spin cartridge. The ssRNA binds to the silica-based membrane in the cartridge, and the digested DNA, free NTPs, and denatured proteins flow through the cartridge.
  3. Wash the membrane-bound ssRNA to eliminate residual RNA Binding Buffer and any remaining impurities.
  4. Elute the ssRNA from the RNA spin cartridge with water.

## Advance Preparation

- [0463] Before using the BLOCK-iT™ RNA Purification reagents for the first time, add 10 ml of 100% ethanol to the entire amount of 5X RNA Wash Buffer to generate a 1X RNA Wash Buffer (total volume = 12.5 ml). Place a check in the box on the 5X RNA Wash Buffer label to indicate that the ethanol was added. Store the 1X RNA Wash Buffer at room temperature.

- [0464] The RNA Binding Buffer contains guanidine isothiocyanate. This chemical is harmful if it comes in contact with the skin or is inhaled or swallowed. Always wear a laboratory coat, disposable gloves, and goggles when handling solutions containing this chemical.

- [0465] Do not add bleach or acidic solutions directly to solutions containing guanidine isothiocyanate or sample preparation waste. Guanidine isothiocyanate forms reactive compounds and toxic gases when mixed with bleach or acids.

#### Materials Needed

- [0466] Have the following materials on hand before beginning:
- RNA transcription reactions (from Step 4; for each gene, you should have a sense transcription reaction and an antisense transcription reaction)
  - RNA Binding Buffer (supplied with the kit, Box 3)
  - $\beta$ -mercaptoethanol
  - 100% ethanol
  - RNA spin cartridges (supplied with the kit, Box 3; one for each sample)
  - 1X RNA Wash Buffer (see Advance Preparation, above)
  - RNase-Free Water (supplied with the kit, Box 3)
  - RNA Recovery Tubes (supplied with the kit, Box 3; one for each sample)
  - 50X RNA Annealing Buffer (supplied with the kit, Box 3)

#### ssRNA Purification Procedure

- [0467] Use this procedure to purify ssRNA produced in the transcription reaction, Step 4.
- [0468] Immediately before beginning, remove the amount of RNA Binding Buffer needed and add  $\beta$ -mercaptoethanol to a final concentration of 1% (v/v). Use fresh and discard any unused solution.
1. To each RNA transcription reaction (~40  $\mu$ l volume), add 160  $\mu$ l of RNA Binding Buffer containing 1% (v/v)  $\beta$ -mercaptoethanol followed by 100  $\mu$ l of 100% ethanol to obtain a final volume of 300  $\mu$ l. Mix well by pipetting up and down 5 times.

2. Apply the sample (~300  $\mu$ l) to the RNA Spin Cartridge. Centrifuge at 14,000 x g for 15 seconds at room temperature. Discard the flow-through.
3. Add 500  $\mu$ l of 1X RNA Wash Buffer to the RNA Spin Cartridge containing bound ssRNA. Centrifuge at 14,000 x g for 15 seconds at room temperature. Discard the flow-through.
4. Repeat the wash step (Step 3, above).
5. Centrifuge the RNA Spin Cartridge at 14,000 x g for 1 minute at room temperature to remove residual 1X RNA Wash Buffer from the cartridge and to dry the membrane.
6. Remove the RNA Spin Cartridge from the Wash Tube, and place it in an RNA Recovery Tube.
7. Add 40  $\mu$ l of RNase-Free Water to the RNA Spin Cartridge. Let stand at room temperature for 1 minute, then centrifuge the RNA Spin Cartridge at 14,000 x g for 2 minutes at room temperature to elute the ssRNA.
8. Add 40  $\mu$ l of RNase-Free Water to the RNA Spin Cartridge and repeat Step 7, eluting the ssRNA into the same RNA Recovery Tube. The total volume of eluted ssRNA is 80  $\mu$ l.
9. Depending on your downstream application, perform the following:
  - (a) If you plan to use the purified ssRNA to generate dsRNA for use in RNAi studies, add 1.4  $\mu$ l of 50X RNA Annealing Buffer to the eluate to obtain a final concentration of 1X RNA Annealing Buffer. Proceed to Determining the RNA Concentration, or to Step 10.
  - (b) If you plan to use the purified ssRNA for applications such as Northern analysis, proceed to Step 10.
10. Store the purified ssRNA at -80°C.

### Determining the ssRNA Purity and Concentration

[0469] Follow the guidelines below to determine the purity and concentration of your purified ssRNA.

1. Dilute an aliquot of the purified ssRNA 100-fold into 1X RNA Annealing Buffer in a total volume appropriate for your quartz cuvette and spectrophotometer.
2. Measure OD at A260 and A280 in a spectrophotometer. Blank the sample against 1X RNA Annealing Buffer.
3. Calculate the concentration of the ssRNA by using the following equation:  
$$\text{ssRNA concentration } (\mu\text{g/ml}) = \text{A260} \times \text{Dilution factor (100)} \times 40 \mu\text{g/ml}.$$
4. Calculate the yield of the ssRNA by using the following equation:  
$$\text{ssRNA yield } (\mu\text{g}) = \text{ssRNA concentration } (\mu\text{g/ml}) \times \text{volume of ssRNA (ml)}$$
5. Evaluate the purity of the purified ssRNA by determining the A260/A280 ratio. For optimal purity, the A260/A280 ratio should range from 1.9-2.2.

### How Much ssRNA to Expect

[0470] The typical yield of purified ssRNA obtained from a 1 kb DNA template ranges from 50-80  $\mu\text{g}$  in a 40  $\mu\text{l}$  transcription reaction. However, yields may vary depending on the size of the DNA template and its sequence. Generally, ssRNA yields are lower for DNA templates smaller than 500 bp or larger than 1 kb.

[0471] After purification, we recommend saving an aliquot of your sense and antisense ssRNA samples for gel analysis. We generally verify the integrity of the dsRNA sample (after annealing) and compare it to the sense and antisense ssRNA samples using agarose or polyacrylamide gel electrophoresis.

[0472] If you wish to verify the integrity of your sense and antisense ssRNA samples before annealing, we suggest running a small aliquot of each sample on a 6% Novex® TBE-Urea Gel (Invitrogen, Catalog no. EC68652BOX), and



including the 0.16-1.77 kb RNA Ladder (Invitrogen, Catalog no. 15623-010) as a molecular weight standard.

#### Generating dsRNA

[0473] To generate dsRNA, you will anneal equal amounts of the purified sense and antisense transcripts of your gene of interest (from ssRNA Purification Procedure, Step 8). Guidelines and instructions are provided below.

#### Amount of ssRNA to Anneal

[0474] You may anneal any amount of sense and antisense transcripts to generate dsRNA; however, use equal amounts of each transcript for optimal results. We generally anneal 50-80  $\mu\text{g}$  of ssRNA to generate 100-160  $\mu\text{g}$  of dsRNA, respectively (e.g. annealing 50  $\mu\text{g}$  of sense transcripts and 50  $\mu\text{g}$  of antisense transcripts results in 100  $\mu\text{g}$  of dsRNA). You may assume that the annealing step is nearly 100% efficient. You will need to know the concentration of each ssRNA before beginning.

#### Materials Needed

[0475] Have the following materials on hand before beginning.

- Purified sense transcripts of your gene of interest
- Purified antisense transcripts of your gene of interest
- 50X RNA Annealing Buffer (supplied with the kit, Box 3)
- 0.5 ml sterile, RNase-free microcentrifuge tube
- 500 ml glass beaker

#### Annealing Procedure

[0476] Use the procedure below to anneal sense and antisense transcripts to generate dsRNA. Remember to use RNase-free supplies and wear gloves to prevent RNase contamination.

1. In a sterile, RNase-free microcentrifuge tube, mix equal amounts of purified sense and antisense transcripts. Place the tube on ice.
2. Heat approximately 250 ml of water to boiling in a 500 ml glass beaker.
3. Remove the beaker of water from the hot plate or microwave and set on your laboratory bench.
4. Place the tube containing the mixture of sense and antisense transcripts in a tube float or a rack in the glass beaker.
5. Allow the water to cool to room temperature for 1-1.5 hours. The ssRNAs will anneal during this time.
6. Remove a small aliquot of dsRNA and analyze by agarose or polyacrylamide gel electrophoresis to check the quality of your dsRNA.
7. Store the dsRNA at -80°C. Depending on the amount of dsRNA produced and your downstream application, you may want to aliquot the dsRNA before storage at -80°C.

[0477] When using the dsRNA, avoid repeated freezing and thawing as dsRNA can degrade with each freeze/thaw cycle.

#### Alternative Annealing Procedure

[0478] If you want to generate dsRNA more quickly, use the alternative annealing procedure below. Note however, that this method is less efficient and will result in lower yields of dsRNA than the slow-annealing method described above.

1. In a sterile, RNase-free microcentrifuge tube, mix equal amounts of purified sense and antisense transcripts.
2. Place the tube in a 75°C heat block for 5 minutes.
3. Remove the tube from the heat block and place in a rack at room temperature for 5 minutes. The ssRNAs will anneal during this time.

4. Remove a small aliquot of dsRNA and analyze by agarose or polyacrylamide gel electrophoresis to check the quality of your dsRNA (see below).
5. Store the dsRNA at -80°C. Depending on the amount of dsRNA produced and your downstream application, you may want to aliquot the dsRNA before storage at -80°C.

[0479] When using the dsRNA, avoid repeated freezing and thawing as dsRNA can degrade with each freeze/thaw cycle.

#### Checking the Integrity of dsRNA

[0480] You may verify the integrity of your dsRNA using agarose or polyacrylamide gel electrophoresis, if desired. We suggest running a small aliquot of your annealing reaction (equivalent to 100-200 ng of dsRNA) on the appropriate gel and comparing it to an aliquot (100-200 ng) of your starting sense and antisense ssRNA. Be sure to include an appropriate molecular weight standard. We generally use the following gels and molecular weight standard:

Agarose gel: 1.2% agarose-TAE gel

Polyacrylamide gel: 6% Novex® TBE Gel (Invitrogen, Catalog no. EC6265BOX)

Molecular weight standard: 0.16-1.77 kb RNA Ladder (Invitrogen, Catalog no. 15623-010)

#### What You Should See

[0481] When analyzing the annealing reaction (see above) using gel electrophoresis, we generally observe a predominant band corresponding to the dsRNA (see Figure 16). If you have used one of the recommended annealing procedures (see above), no ssRNA molecules should be detected.

[0482] A high molecular weight smear is often visible in the annealed samples. This is generally due to branched annealing that occurs when

multiple overlapping ssRNA anneal to each other. These products can be diced *in vitro* or *in vivo* to generate siRNA.

#### Example of Expected Results

[0483] In this experiment, dsRNA representing a 730 bp region of the green fluorescent protein (GFP) gene and a 1 kb region of the luciferase gene were generated using the reagents supplied in the kit and following the recommended protocols in the manual. One microgram of each dsRNA was analyzed on a 1.2% agarose-TAE gel and compared to 0.5 µg of each corresponding purified sense and antisense ssRNA (non-denatured).

[0484] Results are shown in Figure 16: The annealed GFP (lane 4) and luciferase (lane 7) dsRNA samples both show a predominant band that differs in size from each component sense and antisense ssRNA. No ssRNA is visible in the annealed sample. A high molecular weight smear due to branched annealing products is also visible in the annealed samples (lanes 4 and 7).

[0485] In some cases, multiple bands due to secondary structure are observed in the ssRNA samples (e.g., lanes 5 and 6). This is a result of analysis on non-denaturing agarose gels.

#### What to Do Next

[0486] Once you have obtained dsRNA, you have the following options:

1. Use the dsRNA directly to perform RNAi studies in invertebrate systems. Depending on the invertebrate system chosen (e.g. *C. elegans*, *Drosophila*, trypanosomes), multiple methods may exist to introduce the dsRNA into the organism or cell line of choice including injection, soaking in media containing dsRNA, or transfection. Choose the method best suited for your invertebrate system.
2. Use the dsRNA in an *in vitro* reaction with the Dicer enzyme to generate d-siRNA. The resulting d-siRNA may then be transfected into mammalian cells for RNAi studies. For optimized reagents and protocols to generate highly pure d-siRNA from a dsRNA substrate

using recombinant human Dicer enzyme, and to efficiently transfect the d-siRNA into a mammalian cell line of interest using Lipofectamine™ 2000 Reagent, we recommend using the BLOCK-iT™ Dicer RNAi Transfection Kit (Catalog no. K3600-01) or the BLOCK-iT™ Complete Dicer RNAi Kit (Catalog no. K3650-01) available from Invitrogen. For detailed instructions to perform the dicing and transfection reactions, refer to the BLOCK-iT™ Dicer RNAi Kits manual.

### Troubleshooting

[0487] Review the information in this section to troubleshoot the amplification, TOPO® Linking, transcription, and purification procedures.

### Amplifying the Gene of Interest

[0488] The table below lists some potential problems and possible solutions that may help you troubleshoot your amplification reactions.

Problem	Reason	Solution
No PCR product	Poor quality of DNA template	Prepare new template DNA and verify the integrity of the DNA before amplification.
	Poor quality PCR reagents or inactive thermostable DNA polymerase	Amplify the control vector using the primers supplied with the kit and the protocol above. If no PCR product is produced, use fresh PCR reagents and thermostable DNA polymerase.
	Suboptimal PCR conditions	<ul style="list-style-type: none"> <li>• Check the <math>T_m</math> of the PCR primers and adjust your cycling conditions.</li> <li>• Optimize PCR conditions. Refer to the manufacturer's recommendations for your polymerase.</li> </ul>
Low yield of PCR product	Suboptimal PCR conditions	Optimize PCR conditions. Refer to the manufacturer's recommendations for your polymerase.
	Used old DNA polymerase	Use fresh thermostable DNA polymerase.

Problem	Reason	Solution
	Not enough PCR cycles performed	Increase the number of PCR cycles.
Multiple non-specific bands or smearing observed on agarose gel	Suboptimal cycling conditions	Optimize PCR conditions. Refer to the manufacturer's recommendations for your polymerase.
	DNA template contaminated with other DNA	Prepare new template DNA and verify the integrity of the DNA before amplification.
	Poor quality PCR primers	Use HPLC-purified primers to produce your PCR product.

### TOPO® Linking and Secondary Amplification

[0489] The table below lists some potential problems and possible solutions that you may use to help you troubleshoot the TOPO® Linking reaction and the secondary amplification reactions.

Problem	Reason	Solution
No linear DNA template(s) of the expected size obtained	Inefficient TOPO® Linking <ul style="list-style-type: none"> <li>Incubated the TOPO® Linking reaction at 37°C for too long</li> <li>Used a proofreading polymerase to generate the primary PCR product</li> </ul>	<ul style="list-style-type: none"> <li>Do not incubate the TOPO® Linking reaction at 37°C for longer than 15 minutes.</li> <li>Use <i>Taq</i> polymerase (e.g. Platinum® <i>Taq</i>) to generate the primary PCR product. Alternatively, add 3' A-overhangs to the PCR product (see procedure above).</li> </ul>
	Poor quality PCR reagents or inactive thermostable DNA polymerase	Use fresh PCR reagents and thermostable DNA polymerase for the secondary amplification reactions.
	Primers used to produce the primary PCR product contained 5' phosphates	Do not add 5' phosphates to the primers used to produce the primary PCR product.

Problem	Reason	Solution
	TOPO® Linking reaction stored incorrectly	For optimal results, perform secondary amplification reactions directly after TOPO® Linking. If desired, store the TOPO® Linking reaction at -20°C overnight.
Low yield of linear DNA template obtained	Inefficient TOPO® Linking <ul style="list-style-type: none"> <li>Primary PCR product was too dilute</li> <li>Primary PCR product was not fresh</li> <li><i>Taq</i> polymerase and proofreading polymerase mixture used to generate primary PCR product</li> </ul>	<ul style="list-style-type: none"> <li>Purify and concentrate the PCR product using the procedure above.</li> <li>For optimal results, use fresh PCR product in the TOPO® Linking reaction.</li> <li>Use <i>Taq</i> polymerase to generate the primary PCR product or use the procedure above to add 3' A-over-hangs to the PCR product prior to TOPO® Linking.</li> </ul>
	Annealing temperature was too high	Check the $T_m$ s of your PCR primers. Reduce the annealing temperature.
	$T_m$ of the gene-specific primer(s) not compatible with the $T_m$ of the BLOCK-iT™ T7 Primer	Re-design the gene-specific primer(s), making sure that the $T_m$ of each primer is compatible with the $T_m$ of the BLOCK-iT™ T7 Primer.
	Not enough PCR cycles performed	Increase the number of PCR cycles.

### Transcribing and Purifying ssRNA

[0490] The table below lists some potential problems and possible solutions that may help you troubleshoot the transcription and purification steps.

Problem	Reason	Solution
Low ssRNA yield	No ethanol or RNA Binding Buffer added to the sample	Add RNA Binding Buffer containing 1% (v/v) $\beta$ -mercaptoethanol followed by 100% ethanol to the sample (see ssRNA Purification Procedure, Step 1, above).

Problem	Reason	Solution
	Linear DNA template too dilute	<ul style="list-style-type: none"> <li>Purify and concentrate the linear DNA template using the procedure on above.</li> <li>Extend the incubation time of the trans-cription reaction up to 6 hours at 37°C.</li> </ul>
	Transcription reaction not incubated long enough	Extend the incubation time of the transcription reaction up to 6 hours at 37°C.
	Eluted ssRNA from the RNA Spin Cartridge using buffer, not water	Elute ssRNA from the RNA Spin Cartridge using RNase-free water.
	Concentration of ssRNA incorrectly determined <ul style="list-style-type: none"> <li>Sample diluted into water for spectrophotometry</li> <li>Sample blanked against water</li> </ul>	<ul style="list-style-type: none"> <li>Dilute sample in 1X RNA Annealing Buffer for spectrophotometry.</li> <li>Blank sample against 1X RNA Annealing Buffer.</li> </ul>
No ssRNA obtained	Sample contaminated with RNase	<ul style="list-style-type: none"> <li>Use RNase-free reagents and supplies.</li> <li>Wear gloves when handling RNA-containing samples.</li> </ul>
	Gene-specific primers used to amplify TOPO® Linked products, not the BLOCK-iT™ T7 Primer	Use the BLOCK-iT™ T7 Primer and the gene-specific forward or reverse primer in the secondary amplification reaction to generate sense and antisense DNA templates, respectively.
	Forgot to add ethanol to the 5X RNA Wash Buffer	Add 10 ml of ethanol to the 5X RNA Wash Buffer (2.5 ml) to obtain a 1X RNA Wash Buffer.
Volume of eluted ssRNA is > 80 µl	RNA Spin Cartridge containing bound ssRNA not centrifuged to remove residual 1X RNA Wash Buffer	Centrifuge RNA Spin Cartridge at 14,000 × g for 1 minute at room temperature to remove residual 1X RNA Wash Buffer and to dry the membrane (see Step 5, above).  Contamination of eluted ssRNA with 1X RNA Wash Buffer or other impurities can result in inaccurate quantitation of ssRNA, potential toxic effects on invertebrate cells, or reduced dicing efficiency.



Problem	Reason	Solution
A260/A280 ratio not in the 1.9-2.2 range	Sample was not washed with 1X RNA Wash Buffer	Wash the RNA Spin Cartridge containing bound ssRNA twice with 1X RNA Wash Buffer (see Steps 3 and 4, above).
	RNA Spin Cartridge containing bound ssRNA not centrifuged to remove residual 1X RNA Wash Buffer	Centrifuge RNA Spin Cartridge at 14,000 x g for 1 minute at room temperature to remove residual 1X RNA Wash Buffer and to dry the membrane (see Step 5, above).

### RNAi Analysis

[0491] The table below lists some potential problems and possible solutions that may help you troubleshoot your RNAi analysis using dsRNA.

Problem	Reason	Solution
Low levels of gene knockdown observed	dsRNA was degraded <ul style="list-style-type: none"> <li>dsRNA was not stored in 1X RNA Annealing Buffer</li> <li>dsRNA was frozen and thawed multiple times</li> </ul>	<ul style="list-style-type: none"> <li>Be sure to store the dsRNA in 1X RNA Annealing Buffer.</li> <li>Aliquot dsRNA and avoid repeated freeze/thaw cycles.</li> </ul>
No gene knockdown observed	Target sequence contains no active siRNA	Select a larger target region or a different target sequence.
	dsRNA contaminated with RNase	<ul style="list-style-type: none"> <li>Use RNase-free reagents and supplies.</li> <li>Wear gloves when handling RNA-containing samples.</li> </ul>
Non-specific gene knockdown effects observed	Target sequence contains strong homology to other genes	<ul style="list-style-type: none"> <li>Select a new target sequence.</li> <li>Limit the size range of the target sequence to 1 kb.</li> </ul>

### Performing the Control Reactions

[0492] We recommend performing the following control reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves the following steps:

1. Producing a control PCR product using the pcDNA<sup>TM</sup>1.2/V5-GW/lacZ control plasmid and the LacZ Forward 2 and LacZ Reverse 2 primers supplied with the kit.
2. Performing a TOPO® Linking reaction with the control PCR product and the BLOCK-iT<sup>TM</sup> T7-TOPO® Linker.
3. Performing two secondary amplification reactions with the TOPO® Linked PCR product to produce sense and antisense control DNA templates.
4. Using the control DNA templates in transcription reactions to generate sense and antisense RNA transcripts.
5. Purifying the sense and antisense RNA transcripts, and annealing the ssRNAs to produce control dsRNA.

Producing the Control PCR Product

[0493] Use this procedure to amplify the pcDNA<sup>TM</sup>1.2/V5-GW/lacZ control plasmid using Platinum® Taq polymerase. If you are using another thermostable DNA polymerase, follow the manufacturer's instructions to set up the PCR reaction.

1. To produce the 1 kb control PCR product, set up the following 50 µl PCR:

pcDNA <sup>TM</sup> 1.2/V5-GW/lacZ (10 ng/µl)	1 µl
10X PCR Buffer	5 µl
40 mM dNTPs	1 µl
LacZ forward 2 primer (65 ng/µl)	1 µl
LacZ reverse 2 primer (65 ng/µl)	1 µl
Sterile Water	40.5 µl
<u>Platinum® Taq Polymerase (5 U/µl)</u>	<u>0.5 µl</u>
Total Volume	50 µl

2. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	15 seconds	94°C	30X
Annealing	30 seconds	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

3. Remove 1-5  $\mu\text{l}$  from the reaction and analyze by agarose gel electrophoresis. A discrete 1 kb band should be visible.

#### Control TOPO® Linking Reaction

[0494] Using the control PCR product produced in Step 3, above and the BLOCK-iT™ T7-TOPO® Linker, set up the TOPO® Linking reaction as described below.

1. Set up the following control TOPO® Linking reaction:

Control PCR product	1 $\mu\text{l}$
Salt Solution	1 $\mu\text{l}$
Sterile water	3 $\mu\text{l}$
BLOCK-iT™ T7-TOPO® Linker	1 $\mu\text{l}$
Total volume	6 $\mu\text{l}$

2. Incubate at 37°C for 15 minutes and place on ice.
3. Proceed directly to the Secondary Control PCR Reactions, below.

#### Secondary Control PCR Reactions

[0495] Use this procedure to amplify the TOPO® Linked control PCR product using Platinum® Taq polymerase to generate sense and antisense control DNA templates. If you are using another thermostable DNA polymerase, follow the manufacturer's instructions to set up the PCR reaction.

1. Set up the following 50  $\mu\text{l}$  PCR reactions:

Reagent	Sense Template	Antisense Template
Control TOPO® Linking Reaction	1 $\mu\text{l}$	1 $\mu\text{l}$
10X PCR Buffer	5 $\mu\text{l}$	5 $\mu\text{l}$
40 mM dNTPs	1 $\mu\text{l}$	1 $\mu\text{l}$
BLOCK-iT™ T7 Primer (75 ng/ $\mu\text{l}$ )	1 $\mu\text{l}$	1 $\mu\text{l}$
LacZ Forward 2 Primer (65 ng/ $\mu\text{l}$ )	—	1 $\mu\text{l}$
LacZ Reverse 2 Primer (65 ng/ $\mu\text{l}$ )	1 $\mu\text{l}$	—
Sterile Water	40.5 $\mu\text{l}$	40.5 $\mu\text{l}$
Platinum® Taq Polymerase (5 U/ $\mu\text{l}$ )	0.5 $\mu\text{l}$	0.5 $\mu\text{l}$
Total volume	50 $\mu\text{l}$	50 $\mu\text{l}$

2. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	15 seconds	94°C	30X
Annealing	30 seconds	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

3. Remove 1-5 µl from the reaction and analyze by agarose gel electrophoresis. A discrete band of approximately 1 kb should be visible.

#### Generating Control dsRNA

[0496] Once you have generated the sense and antisense control DNA templates, you may use these templates in transcription reactions to produce sense and antisense control transcripts. After purification, these transcripts may then be annealed to produce control dsRNA. Follow the protocols above to produce and purify sense and antisense transcripts, and to anneal the purified transcripts to produce dsRNA.

#### What To Do With the Control dsRNA

[0497] The lacZ dsRNA may be used as a control for RNAi analysis in the following ways:

##### Invertebrate Systems:

Use as a negative control for non-specific activity in any invertebrate system.

##### Mammalian Systems:

For some embryonic stem cell (ES) cell lines in which the CMV promoter is active (e.g. AB2.2), you may use the lacZ dsRNA as a positive control for gene knockdown (Yang et al., 2001). Simply introduce the pcDNA<sup>TM</sup>1.2/V5-GW/lacZ reporter plasmid and the lacZ dsRNA into cells and assay for inhibition of  $\beta$ -galactosidase expression.

Alternatively, you may use the lacZ dsRNA in Invitrogen's BLOCK-iT<sup>TM</sup> Dicer RNAi Transfection Kit as a substrate to produce diced short interfering RNA (d-siRNA). The lacZ d-siRNA may then be used as a negative control for non-specific activity in the mammalian cell line of interest or as a positive control for knockdown of  $\beta$ -galactosidase expression from the pcDNA<sup>TM</sup>1.2/V5-GW/lacZ reporter plasmid. For detailed instructions to produce d-siRNA, refer to the BLOCK-iT<sup>TM</sup> Dicer RNAi Kits manual.

#### Gel Purifying PCR Products

[0498] Smearing, multiple banding, primer-dimer artifacts, or large PCR products (> 1 kb) may necessitate gel purification. If you intend to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to Current Protocols in Molecular Biology, Unit 2.6 (Ausubel et al., 1994) for the most common protocols. Two simple protocols are provided below.

#### Using the S.N.A.P.<sup>TM</sup> Gel Purification Kit

[0499] The S.N.A.P.<sup>TM</sup> Gel Purification Kit (Catalog no. K1999-25) allows you to rapidly purify PCR products from regular agarose gels.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel.

Do not use TBE to prepare agarose gels. Borate will interfere with the sodium iodide step, below.

2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of 6 M sodium iodide solution.

Add 1.5 volumes of Binding Buffer.

3. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.<sup>™</sup> column. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.
4. If you have solution remaining from Step 3, repeat Step 4.
5. Add 900 µl of the Final Wash Buffer.
6. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
7. Repeat Step 7.
8. Elute the purified PCR product in 30 µl of sterile water. Use 1 µl for the TOPO® Linking reaction and proceed as described above.

#### Quick S.N.A.P.<sup>™</sup> Method

**[0500]** An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.<sup>™</sup> column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO® Linking reaction. Be sure to make the gel slice as small as possible for best results.

#### Adding 3' A-Overhangs Post-Amplification

**[0501]** Direct TOPO® Linking of DNA amplified by proofreading polymerases with the BLOCK-iT<sup>™</sup> T7-TOPO® Linker is difficult because of very low TOPO® Linking efficiencies. These low efficiencies are caused by the 3' to 5' exonuclease activity associated with proofreading polymerases which removes the 3' A-overhangs necessary for TA Cloning®. A simple method is provided below to clone these blunt-ended fragments.

### Before Starting

[0502] You will need the following items:

Taq polymerase

A heat block equilibrated to 72°C

Phenol-chloroform (optional)

3 M sodium acetate (optional)

100% ethanol (optional)

80% ethanol (optional)

TE buffer (optional)

### Procedure

[0503] This is just one method for adding 3' adenines. Other protocols may be suitable.

1. After amplification with Vent® or Pfu polymerase, place vials on ice and add 0.7-1 unit of Taq polymerase per tube. Mix well. It is not necessary to change the buffer.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Place the vials on ice. Proceed to TOPO® Linking (see above).

[0504] If you plan to store your sample(s) overnight before proceeding with TOPO® Linking, you may want to extract your sample(s) with phenol-chloroform to remove the polymerases. After phenol-chloroform extraction, precipitate the DNA with ethanol and resuspend the DNA in TE buffer to the starting volume of the amplification reaction.

### Purifying and Concentrating PCR Products

[0505] If your gene of interest has not amplified efficiently and the yield of your PCR product is low, you may use the S.N.A.P.™ MiniPrep Kit available from Invitrogen (Catalog no. K1900-25) to rapidly purify and concentrate the PCR product. Other resin-based purification kits are suitable.

### Materials Needed

- [0506] You should have the following reagents on hand before beginning:
- Isopropanol
  - Binding Buffer (supplied with the S.N.A.P.<sup>™</sup> MiniPrep Kit )
  - Wash Buffer (supplied with the S.N.A.P.<sup>™</sup> MiniPrep Kit)
  - Final Wash Buffer (supplied with the S.N.A.P.<sup>™</sup> MiniPrep Kit)
  - Sterile water
  - S.N.A.P.<sup>™</sup> MiniPrep columns (supplied with the S.N.A.P.<sup>™</sup> MiniPrep Kit)

### Purification Protocol

- [0507] Follow the protocol below to purify your PCR product using the S.N.A.P.<sup>™</sup> MiniPrep Kit. The protocol provides instructions to purify PCR products from a 50 µl reaction volume. To purify PCR products from larger reaction volumes (e.g. several PCR reactions pooled together), scale up the volumes of each buffer accordingly. Details about the components of the S.N.A.P.<sup>™</sup> MiniPrep Kit can be found in the S.N.A.P.<sup>™</sup> MiniPrep Kit manual.

1. Add 150 µl of Binding Buffer to the 50 µl PCR reaction. Mix well by pipetting up and down.
2. Add 350 µl of isopropanol. Mix well by vortexing.
3. Immediately load solution from Step 2 onto a S.N.A.P.<sup>™</sup> MiniPrep column. Centrifuge for 30 seconds at 1000 x g in a microcentrifuge and discard the flow-through.
4. Add 250 µl of the Wash Buffer and centrifuge for 30 seconds at 1000 x g in a microcentrifuge. Discard the flow-through.
5. Add 450 µl of the Final Wash Buffer and centrifuge for 30 seconds at 1000 x g in a microcentrifuge. Discard the flow-through.
6. Centrifuge for an additional 30 seconds at full-speed in a microcentrifuge to dry the column.



7. Transfer the column to a new collection tube. Add 30 µl of sterile water to the column. Incubate at room temperature for 1 minute.
8. Centrifuge for 30 seconds at full-speed in a microcentrifuge to elute the DNA. Collect the flow-through. Use 1 µl in the TOPO® Linking reaction (see above).

**[0508]** pcDNA™1.2/V5-GW/lacZ (6498 bp) (see Figure 17) is a control vector expressing a C-terminally-tagged β-galactosidase fusion protein under the control of the human cytomegalovirus (CMV) promoter (Andersson et al., 1989; Boshart et al., 1985; Nelson et al., 1987), and was generated using the MultiSite Gateway® Three-Fragment Vector Construction Kit available from Invitrogen (Catalog no. 12537-023). Briefly, a MultiSite Gateway® LR recombination reaction was performed with pDEST™R4-R3 and entry clones containing the CMV promoter, lacZ gene, and V5 epitope and TK polyadenylation signal to generate the pcDNA™1.2/V5-GW/lacZ vector. β-galactosidase is expressed as a C-terminal V5 fusion protein with a molecular weight of approximately 119 kDa. The complete sequence of pcDNA™1.2/V5-GW/lacZ is available from Invitrogen.

#### Product Qualification

**[0509]** This section describes the criteria used to qualify the components of the BLOCK-iT™ RNAi TOPO® Transcription Kit.

#### Functional Qualification

**[0510]** The components of the BLOCK-iT™ RNAi TOPO® Transcription Kit are functionally qualified as follows:

1. Using the pcDNA™1.2/V5-GW/lacZ plasmid and the LacZ Forward 2 and LacZ Reverse 2 primers supplied with the kit, a control PCR product is generated and TOPO® Linked to the BLOCK-iT™ T7-TOPO® Linker following the protocols above.
2. Using the BLOCK-iT™ T7 Primer and the LacZ Forward 2 or LacZ Reverse 2 primer, two aliquots of the TOPO® Linking reaction are

amplified following the procedure above to generate sense and antisense DNA templates. An aliquot of each secondary PCR reaction is analyzed on an agarose gel and compared to an aliquot of the primary PCR product. The sense and antisense DNA template should demonstrate a gel shift (1043 bp) when compared to the primary PCR product (1000 bp).

3. The sense and antisense DNA templates are transcribed using the reagents supplied in the kit and following the procedure above. The sense and antisense transcripts are analyzed on a 6% Novex® TBE-Urea Gel (Invitrogen, Catalog no. EC68652BOX). The 0.16-1.77 kb RNA Ladder (Invitrogen, Catalog no. 15623-010) is included as a molecular weight standard. RNA should be visible in the lanes containing sense and antisense transcripts, while no RNA should be observed from a transcription reaction using a template generated from a PCR product that was not linked to the BLOCK-iT™ T7 Linker.
4. The sense and antisense transcripts are purified using the reagents supplied in the kit and following the procedure above. Following purification, the purified sense and antisense ssRNA are quantitated using spectrophotometry. Each transcription reaction should yield at least 60 µg of ssRNA, and the A260/A280 ratio should be between 1.9 and 2.2.
5. Equal amounts of sense and antisense RNA are annealed following the procedure above. The dsRNA is analyzed on a 6% Novex® TBE Gel (Invitrogen, Catalog no. EC6265BOX) with the 0.16-1.77 kb RNA Ladder included as a molecular weight standard. A gel shift representing dsRNA should be observed in the annealed sample when compared to sense or antisense ssRNA.

#### pcDNA<sup>TM</sup>1.2/V5-GW/lacZ Plasmid

- [0511] The pcDNA<sup>TM</sup>1.2/V5-GW/lacZ plasmid is qualified by restriction analysis. Restriction digest should demonstrate the correct banding pattern when electrophoresed on an agarose gel.

#### PCR Primers

- [0512] The BLOCK-iT<sup>TM</sup> T7, LacZ Forward 2, and LacZ Reverse 2 primers are functionally qualified by performing the control PCR reactions described on pages above.

#### References

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## Example 11

### Small Nucleic Acids Purification System

[0549] All catalog numbers provided below correspond to Invitrogen Corporation products, Carlsbad, CA, unless otherwise noted.

[0550] Small nucleic acid molecules, especially siRNA, is getting great attention with function in gene specific knockout or silencing of gene expression. Recently, many researchers demonstrated that gene specific siRNA can be generated *in vitro* via a combination of transcription and a ribonuclease enzyme. The digestion of long transcripts is accomplished with a ribonuclease called RNase III or Dicer and the digested sample is required to be purified from the non-processed template, intermediate and buffer component of enzyme reaction. If residual long dsRNA template and other intermediates remained in the sample and were transfected along with siRNA into cells, it might lead to non-specific response. Thus removal of this residual template and intermediate is required for accurate functional analysis of the gene specific siRNA. Pre-existing total RNA purification systems are not suitable and not designed to purify less than 30bp nucleic acids and only a size exclusion spin column has been utilized to select small size nucleic acid from mixtures.

We have developed a buffer formulation to purify dsRNA that is smaller than 30 bp using our pre-existing glass fiber filter. Both single-column and double-column method were developed to purify siRNAs generated using Dicer and RNase III. The purified siRNA can be used to assay cellular functional via gene specific knock out without non-specific interference by >30 bp dsRNA (complete buffer exchange; eluted in DEPC treated H<sub>2</sub>O). This purification procedure can be utilized for other applications such as linker, aptamer, protein binding domain extraction, etc.

## Introduction

[0551] Total RNA is composed of three main transcript categories. These are ribosomal RNAs (28S, 18S, and 5S in the case of mammalian cells), mRNA, and low molecular weight RNA species such as tRNA, snRNA, and others. The recent discovery and rudimentary elucidation of the mechanism of action of RNA interference and the identification of a new regulatory RNA termed short interfering RNA (siRNA) as well as micro RNA are receiving increasing attention by the scientific community. This increased interest is based on siRNA's ability to mediate down-regulation of gene expression by sequence specific, and hence gene specific, degradation of targeted mRNA. The popularity of the siRNA approach is justified as it has distinct advantages over anti-sense methods and knockout approaches. It appears that the siRNA approach is capable of down-regulating gene expression with higher efficiency and efficacy than the antisense approach and offers greater flexibility and ease of use compared to knockout approaches.

[0552] RNA interference is a cellular defense mechanism where a long double-stranded RNA molecule is processed by an endogenous (endo)ribonuclease resulting in the production of small interfering RNAs (siRNAs), which are generally 21 to 23 nucleotides in length. The siRNA molecules bind to a protein complex, RNA Induced Silencing Complex (RISC), which contains a helicase activity that unwinds siRNA molecules, allowing the anti-sense strand of siRNA to bind to complementary mRNA, thus triggering targeted mRNA degradation by endonucleases or blocking mRNA translation into protein (for a review see Denli and Hannon, 2003, Carrington and Ambros, 2003). In addition, siRNA does not trigger an immune response, because it is a natural cellular mechanism (Sledz *et. al.*, 2003)

[0553] Initial attempts of gene specific knockdown using long dsRNA transcripts failed in mammalian cells because of activation of protein kinase PKR and 2', 5'-oligoadenylate synthetase that trigger non-specific shutdown of



protein synthesis and non-specific degradation of mRNA. Elbashir and co-workers demonstrated that transfection of chemically synthesized 21-23 nt dsRNA fragments could specifically suppress gene expression without triggering non-specific gene silencing effects in mammalian cells. However, different suppression levels are often observed with synthetic short siRNAs as they target a single specific site. Under these conditions site accessibility becomes an issue as mRNA containing high levels of secondary or tertiary structure may prevent siRNA/target/RISC complex formation and affect efficacy of the siRNA used. Thus, multiple double-stranded siRNA molecules, usually 4 – 5, need to be screened that target different sequences in a targeted mRNA to identify one siRNA construct with adequate potency for gene suppression in a given mRNA. Short interfering RNA constructs can also be generated by transcription *in vitro* from short DNA templates or by transcription *in vivo* from a transfected DNA construct. However, none of the latter methods are easily scaled up for multiple gene screens due to high cost of oligonucleotides and/or difficulties of target region selection. A new method was recently developed to generate gene specific functional siRNA pools using a combination of RNA transcription followed by digestion with Dicer enzyme. This method generates multiple functional siRNAs from long dsRNA target sequences which correspond to the gene transcript of interest. With this new method, low cost and highly efficient screening of gene knockdown effects is possible and high throughput screening of multiple genes can be achieved. However, the latter methodology requires purification of functional siRNA after digestion of long dsRNA substrate with Dicer. Undigested, long dsRNA substrates as well as intermediate digestion products longer than approximately 30 bp elicit non-specific responses such as non-specific shutdown of translation and initiation of apoptosis (Kaufman, 1999). Others have used size exclusion columns for purification of functional siRNA. However, this purification is not efficient and does not provide high quality siRNA for transfection.

[0554] Our Small Nucleic Acids Purification System provides an efficient means of purification for functional, diced siRNA and other small dsRNA molecules. The purification is based on glass fiber purification technology. The small nucleic acids purification system eliminates dsRNA that exceeds 30 bp in length and selectively and specifically purifies dsRNA shorter than 30 base pairs. In the case of siRNA, the purified dsRNA is of high quality, highly functional for transcript specific gene suppression, and exhibits no cell toxicity. Currently, Invitrogen Block-iT™ Dicer RNAi Kits provide complete Dicer RNAi transfection kit, include RNAi purification kit, Dicer Enzyme kit, Lipofectamine™ 2000 Reagent and/or TOPO® transcription Kit, as bundle product. Small Nucleic Acids Purification kit is a stand-alone product of the siRNA purification module from Block-iT™ that accommodates not only siRNA purification generated by Dicer and RNase III but also other small nucleic acids applications. The Small Nucleic Acids Purification Kit, as related to the purification of enzymatically-generated siRNA (Dicer & RNaseIII), will generally meet the following criteria: (1) Purified siRNA expected not to contain dsRNA molecules greater than 30 bp in length, (2) Suppression levels observed with purified siRNA will be the same or higher than those observed with synthesized siRNA, (3) Recovery of purified material expected to exceed 80%.

#### Spin Column Purification Kit Components

1. 50 individual spin columns assembled in collection tubes in one bag
2. 50 individual recovery tubes in one bag
3. Binding Buffer (47-6001): 11 mL
4. 5x Wash Buffer (47-6003): 15 mL, EtOH (95–100%) added by end user
5. Elution Buffer (47-6002): 3 mL, 1.5 mL EtOH (95–100%) and 1.5 mL RNase-free water to be added by end user
6. DEPC water (47-0005): 10 mL
7. Manual

8. QRC

[0555] The components provided in the kit are sufficient for 50 purifications using the single-column protocol, in which a final ethanol precipitation step in the presence of glycogen as a co-precipitant is desired. The components provided in the kit are sufficient for 25 purifications when using the two-column protocol, in which the second column is used for selective binding of the short target nucleic acids followed by elution in DEPC-treated water to obtain the final, purified product (see Purification Protocol Flowchart)

Optional Materials:

Crude small nucleic acids preparation for purification

Materials for generating long dsRNA template

Materials for digestion of long dsRNA template to generate crude siRNA product

Chemically synthesized siRNA

EtOH (95 or 100%)

UltraPure <sup>TM</sup>Glycogen (20 µg/µl) (Invitrogen cat #10814-010)

Purification Protocol Flowchart

[0556] The Small Nucleic Acids Purification System is designed to purify Micro-RNA molecules such as micro RNA, tiny RNA, small nuclear RNA, guide RNAs, telomerase RNA, small non-mRNA, catalytic RNA, and small regulatory RNAs (such as aptamer). Also, RNAi molecules RNase III-generated diced siRNA (15-16 bp), Dicer-generated siRNA (21-23 bp), other short hairpin RNA, and small temporary regulatory RNA can be purified with the Small Nucleic Acids Purification System.

Single-Column Protocol **	Two-Column Protocol*
Add 150 µl of Binding Buffer to 50 µl of sample reaction volume* and mix it well (Total volume: 200 µl)	Add 50 µl of Binding Buffer to 50 µl of sample reaction volume* and mix it well. (Total volume 100 µl)

Single-Column Protocol **	Two-Column Protocol*
Add 600 µl of EtOH (95-100%) (Final EtOH concentration 71-75%, total volume: 800 µl)	Add 50 µl of EtOH (95-100%) (Final EtOH concentration 31-33%, sample volume: 150 µl)
Mix sample well and load onto spin column	
Centrifuge at 20,000 x g for 1 min	
Expected recovery volume: ca. 750 µl	Expected recovery volume: ca. 130 µl
	Remove spin column from collection tube
	Add 185 µl of EtOH (95 -100%) to pass-through and mix it well. (Final EtOH conc. ca. 70 – 74%)
	Load sample onto 2 <sup>nd</sup> column
	Centrifuge at 20,000 x g for 1 min
Wash spin column with 500 µl of 1X Wash Buffer	
Repeat the washing step (optional)	
Centrifuge at 20,000 x g for 1 min to dry the filter	
Add 100 µl of <b>Elution Buffer</b> to dried spin column and incubate at ambient temperature for 1 min	Add 100 µl of <b>DEPC-treated water</b> to dried spin column & incubate at ambient temperature for 1 min
Centrifuge at 20,000 x g for 1 min	
Expected elution volume: ca. 95 µl	
<b>The eluate contains the purified, short dsRNA</b>	
<b>EtOH precipitation of short nucleic acids:</b> <ol style="list-style-type: none"> <li>Add 200 µl of ice cold 100% EtOH and 1 µl glycogen solution (20 µg/µl).</li> <li>Incubate at –20 °C for 15 min and centrifuge for 15 min at 20,000 x g</li> <li>Discard supernatant carefully and wash pellet with 0.5 ml of 70% EtOH</li> <li>Centrifuge for 10 min at 20,000 x g</li> <li>Discard supernatant and air dry pellet</li> </ol> Resuspend pellet of <b>purified, short dsRNA</b> in 50 µl (or desirable amount) of DEPC-treated water	

\*Higher sample reaction volumes may require proportionally increased Binding Buffer and EtOH volumes. (Two-Column protocol provide here is scaled down procedure from siRNA purification kit module of Block-iT( Dicer RNAi Kit). Either EtOH or isopropanol can be used to mixing step with Binding Buffer.

\*\*Single column purification will limit its reaction volume to 50µl reaction. (up to 10 µg of dsRNA reaction).

[0557] Please see detail description of Purification of Small Nucleic Acids-General Consideration in *Results and Discussion* section.

## Materials and Methods

### Generation of dsRNA and siRNA

[0558] Crude siRNA needed for purifications was generated in a two-step process. First, *in-vitro* T7 RNA polymerase transcription reaction was used to generate the individual strands that form dsRNA, which then, in a second reaction, served as a template for either Dicer or RNase III digestion yielding crude siRNA preparations that were used for purification with the new kit. The genes of LacZ (Accession number: [AY150267](#)) and Luciferase (Accession number: [AAL30778.1](#)) were selected as the target genes for siRNA inhibition. LacZ dsRNA template was generated as follows: (1) PCR was performed with lacZ gene-specific primer 1 (5'-ACC AGA AGC GGT GCC GGA AA -3' (SEQ ID NO: 2)) and primer 2 (5'- CCA CAG GGG ATG GTT CGG AT-3' (SEQ ID NO: 3)), (2) PCR was performed to incorporate T7 sequences at both ends of the amplicon generated in step 1 with Primer 3 (5'- GAC TCG TAA TAC GAC TCA CTA TAG GGA CCA GAA GCG GTG CCG GAA A -3' (SEQ ID NO: 8)) and primer 4 (5'- GAC TCG TAA TAC GAC TCA CTA TAG GGC CAC AGC GGA TGG TTC GGA T-3' (SEQ ID NO: 9)). The resulting amplicon was purified with Qiagen's PCR clean up kit (QIAquick PCR Purification Kit, cat # 28104) and used as template for the T7 RNA polymerase reverse transcription reaction to generate dsRNA. Long dsRNA was treated in a final step before Dicer or RNaseIII digestion with DNase I and RNaseA to remove template DNA and unhybridized single-stranded RNA. Luciferase specific dsRNA was generated analogously using the following primer sets: primer 5 (5'-TGA ACA TTT CGC AGC CTA CC-3' (SEQ ID NO: 4)) and primer 6 (5'- GCC ACC TGA TAT CCT TT- 3' (SEQ ID NO: 10)) for the first round of PCR, primer 7 (5'-GAC TCG TAA TAC GAC TCA CTA TAG GGT GAA CAT TTC GCA GCC TAC C-3' (SEQ ID NO: 11)) and primer 8 (5'-GAC TCG TAA TAC GAC TCA CTA TAG GGG CCA CCT GAT ATC CTT T -3' (SEQ ID NO: 12)) for the

second round of PCR. Plasmids containing the LacZ and Luciferase gene used as templates (pcDNA1.2/V5/GW-lacZ and pcDNA5-FRT-luc). These two plasmids were also used for transfection to serve as reporter plasmid for functional testing. The two plasmids used are components of the BLOCK-iT™ Dicer RNAi Kit (Invitrogen, cat. # K3600-01). Double-stranded RNA, which was to serve as template for siRNA generation, was purified using the glass fiber filter columns developed for siRNA purification as well as with Ambion's purification columns and protocol. Purified dsRNA template was digested with either Dicer (Invitrogen) or RNase III (Ambion) to generate functional siRNA. The latter was purified using the single-column as well as the two-column protocol outlined above.

#### Mammalian Cell Culture and Transfection

[0559] For functional testing GripTite™ 293 MSR cells (Invitrogen, cat. # R79507) and FlpIn 293 cells were used. GripTite™ 293 MSR cells were cultured in DMEM containing 4 mM L-glutamine, 10% FBS, and 600 µg/ml geneticin (Invitrogen, cat.# 11811-023). In co-transfection experiments 100 ng of each reporter plasmid (see above) was co-transfected with either unpurified siRNA, purified siRNA, or synthetic siRNA specific for lacZ or for Green Fluorescent Protein (GFP) into 90% confluent GripTite™ 293 cells plated at 2 x 10<sup>5</sup> cells/well. FlpIn 293 cells (FlpIn 293 luc) expressing luciferase from a single integrated copy were used to test luciferase specific siRNA. LacZ activity was also monitored as a control to assess any general, non-specific changes in mRNA expression. FlpIn 293 cells were cultured in DMEM containing 4 mM L-glutamine, 10% FBS, and 100 µg/ml hygromycin B (Invitrogen, cat. #10687-010). Cells were seeded in 24-well plates and grown to 30 – 50% confluence before transfection with siRNA.

### $\beta$ -Galactosidase and Luciferase Assays

[0560] Activity and specificity of siRNA transfected was assessed by monitoring the activity of the reporter gene products luciferase and  $\beta$ -galactosidase. One to two days after transfection the medium was removed from each well of the 24-well plates and replaced with 500  $\mu$ l cold luciferase lysis buffer from Promega (25 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0, 10% v/v glycerol, 0.1% v/v Triton X-100). Plates were then frozen at  $-80^{\circ}\text{C}$  for at least 1 hour. Samples were thawed for 30 min at RT and 50  $\mu$ l (for luciferase assay) or 10  $\mu$ l (for  $\beta$ -galactosidase assay) were transferred to a black 96-well plate. For  $\beta$ -galactosidase, 90  $\mu$ l of Reaction Dilution Buffer containing 1% (v/v) Galacton-Plus® (Applied Biosystems, cat # T1006) was added to each sample and incubated for 30 min at room temperature. Luminescence was measured on a MicroLumat Plus luminometer using Winglow v.1.24 software (EG&G Berthold). For luciferase, either 50  $\mu$ l of Luciferase Assay Reagent (Promega, cat # E1483) or 100  $\mu$ l Accelerator II (Tropix) were injected per well and readings were taken for 5 seconds after a 2-second delay.

#### Other Materials Used

- i. Silencer siRNA Cocktail Kit (Cat. no. 1625, Ambion Inc.)
- ii. RNA purification Column 1 and 2 (Cat. no., T510004, T510005, Gene Therapy Systems, Inc.)
- iii. Yeast tRNA (Cat. no. 15401-011, Invitrogen Inc.)
- iv. E-Gel 4% (Cat. no. G5018-04, Invitrogen Inc.)
- v. 10 bp DNA ladder (Cat. no. 10821-015, Invitrogen Inc.)

## Results and Discussion

### Purification of Small Nucleic Acids – General Considerations

[0561] Commercially available kits for the isolation and purification of double-stranded nucleic acids, RNA as well as DNA, generally do not address the need for purification of short double-stranded nucleic acids. A notable exception is the use of size exclusion filtration technology. However, this technology suffers from several drawbacks (limited automation capabilities, broad cut-off size ranges, low recoveries, etc.) that have limited its use. Short double-stranded nucleic acids shall be defined here as nucleic acids that are shorter than about 100 bp in length. Ribonucleic acids falling into this category include, but are not limited to, RNA species that are described in the literature as tiny RNA, small RNA (sRNA), non-coding RNA (ncRNA), micro-RNA (miRNA), small non mRNA (snmRNA), functional RNA (fRNA), transfer RNA (tRNA), catalytic RNA such as ribozymes, small nucleolar RNA (snRNA), short hairpin RNA (shRNA), small temporally regulated RNA (strRNA), aptamers, and RNAi molecules including without limitation small interfering RNA (siRNA). With recent developments in the field of RNAi/siRNA technology, a particular need for the purification of siRNA from crude enzymatic preparations of siRNA has become apparent. Small interfering RNAs (siRNA) are small dsRNA molecules in the size range of approximately 12 to 25 bp, which can be generated either enzymatically or chemically (Elbashir *et. al.*, 2001). Short deoxyribonucleic acids potentially requiring purification may comprise, but are not limited to, dsDNA molecules such as adapters, linkers, short restriction fragments and PCR products. The purification system described here is based on nucleic acids binding to a glass fiber filter under controlled conditions permitting size-dependant, efficient, high-recovery, high-purity purification of short nucleic acids.

[0562] Two general approaches for the purification of small nucleic acids are outlined in the purification protocol flowchart above pertaining to the



purification of enzymatically-generated siRNA. Using a single-column protocol, all double-stranded nucleic acids exceeding a length of approximately 10 base pairs are bound to the glass fiber matrix during an initial step in the presence of a chaotropic salt, which is contained in the Binding Buffer, and EtOH in excess of 70% (v/v). The binding step is followed by a wash step, which removes non-nucleic acids components from the target nucleic acids bound to the glass fiber matrix. In a third step small nucleic acids are selectively eluted in Elution Buffer containing a controlled amount of EtOH that is specific for the release of the targeted nucleic acids size range. Nucleic acids exceeding the targeted size range will remain bound to the glass fiber filter. In the case of siRNA, either generated by Dicer or RNaseIII digestion of larger dsRNA template molecules, the optimal concentration of EtOH was determined to be 25% (v/v). Use of the single-column protocol for small nucleic acids purification typically employs a final EtOH precipitation step in order to remove chaotropic salts, which are present in the Elution Buffer, followed by resuspension of purified nucleic acids in a buffer of choice.

[0563] In the two-column protocol double-stranded nucleic acids fragments exceeding a length of approximately 30 base pairs are bound to the glass fiber matrix during the initial binding step, while fragments shorter than approximately 30 base pairs are washed through the glass fiber matrix and are recovered in the flow through. This size fractionation is achieved by applying the mixture of nucleic acids fragment of various sizes in a Binding Buffer containing a chaotropic salt and a controlled concentration of EtOH. In the case of siRNA the optimal concentration of EtOH was determined to be approximately 33% (v/v). The size cut-off for flow through of short nucleic acids can be fine tuned by adjusting the relative amount of EtOH contained in the binding solution. Increased EtOH concentrations result in retention of shorter nucleic acid fragments on the glass fiber filter, while decreased EtOH concentrations in the binding solution result in the elution of larger nucleic acids fragments. In a second step the EtOH concentration of the flow through

from the first column containing the small nucleic acids of interest is increased to >70% (v/v) and applied to a second glass fiber filter column. Under these conditions small, double-stranded nucleic acids are bound to the matrix of the second filter column. This second binding step is followed by a wash step, which removes any remaining non-nucleic acid components from the targeted small nucleic acids bound to the glass fiber matrix. In a final step the targeted small nucleic acids are eluted off the glass fiber matrix at low ionic strength with water.

[0564] The single-column and the two-column protocol provide two alternatives for the purification of small nucleic acids molecules. The single-column protocol is more economical as it uses only one filtration step. However, this protocol typically employs a final EtOH precipitation step, which is more time consuming than a filtration step and holds the risk of incomplete nucleic acid precipitation. On the other hand, EtOH precipitation is generally considered to yield a cleaner nucleic acid preparation. The two-column protocol, while more costly per sample purification, is generally faster than the single-column protocol by virtue of avoiding the EtOH precipitation step.

#### Single-Column Protocol: EtOH Fractionation of Crude siRNA

##### Experimental Setup

[0565] Crude lacZ siRNA, which was generated from 1  $\mu$ g of dsRNA template in a 50- $\mu$ l reaction volume according to the procedure outlined above, was mixed with 50  $\mu$ l of Binding Buffer and 100  $\mu$ l of EtOH at various concentrations. Final EtOH concentrations ranged from 5 – 50%. Samples were applied to spin columns, centrifuged, and the flow-through was collected and analyzed on a 4% E-Gel after EtOH precipitation of nucleic acids in the presence of glycogen.

## Results and Discussion

[0566] See Figure 18. Lane 1 in Figure 18 shows a 10-bp DNA ladder for size reference. The 20- and 30-bp fragments are marked. The crude lacZ/Dicer reaction is shown in lane 2. Undigested, 1-kb dsRNA template migrates close to the well. The undigested material generally accounts for a significant portion of the initial starting material after the dicing reaction, i.e. the Dicer reaction does not completely digest dsRNA substrate even after prolonged reaction times. Since the presence of undigested and partially digested dsRNA substrate is incompatible with cell viability, purification is essential. In the case shown, undigested template accounts for more than 50% of the starting material. The dsRNA Dicer reaction product, which has a length of 21-23 bp, migrates between the 20- and 30-bp fragments of the DNA ladder shown in lane 1. Reaction intermediates, partially digested dsRNA template which are apparent as a background smear in the lane, migrate between the undigested template and the siRNA reaction product. At an EtOH concentration of 5% in the Binding Buffer most of the 1-kb dsRNA template as well as shorter dsRNA molecules do not bind to the filter matrix and are consequently recovered in the flow-through (Figure 18, lane 3). Increasing ethanol concentration in the Binding Buffer leads to the binding of progressively shorter dsRNA fragments to the filter matrix resulting in the selective binding of unwanted longer dsRNA fragments and selective flow-through of targeted siRNA molecules (Figure 18, lanes 4 – 12). At an EtOH concentration exceeding 20% it appears that only targeted 21-23 bp siRNA selectively elute while longer dsRNA fragments are retained on the filter. At EtOH concentrations of 20 – 30% (lanes 6 – 8) recovery appears to be efficient, while at higher ethanol concentrations (35 – 50%, lanes 9 – 12) recovery decreases due to binding of even short dsRNA molecules at these elevated EtOH concentrations. At EtOH concentrations exceeding 50% siRNA showed increasing affinity for the glass fiber matrix of the filter. Efficient binding of

siRNA can be achieved with EtOH concentrations of 70% or more even for the shorter siRNA products derived from RNase III digestion (see below).

[0567] Figure 18 shows fractionation of double-stranded RNA using different ethanol concentrations. Flow-through samples were analyzed on a 4% E-Gel after EtOH precipitation in the presence of glycogen and resuspension in RNase-free water.

Lane 1: 10 bp DNA Ladder (Invitrogen Cat # 18021-015)

Lane 2: Crude lacZ/Dicer siRNA reaction with 1-kb dsRNA template

Lane 3: Flow-through of 5% EtOH-containing Binding Buffer

Lane 4: Flow-through of 10% EtOH-containing Binding Buffer

Lane 5: Flow-through of 15% EtOH-containing Binding Buffer

Lane 6: Flow-through of 20% EtOH-containing Binding Buffer

Lane 7: Flow-through of 25% EtOH-containing Binding Buffer

Lane 8: Flow-through of 30% EtOH-containing Binding Buffer

Lane 9: Flow-through of 35% EtOH-containing Binding Buffer

Lane 10: Flow-through of 40% EtOH-containing Binding Buffer

Lane 11: Flow-through of 45% EtOH-containing Binding Buffer

Lane 12: Flow-through of 50% EtOH-containing Binding Buffer

#### Conclusion

[0568] Removal of undigested and partially digested dsRNA substrate and high-purity recovery of Dicer-generated siRNA can be achieved by controlling EtOH concentration in the final binding solution. Optimal results are achieved with final EtOH concentrations ranging from 20 – 30% in the binding solution.

## Functional Testing of Purified siRNA (Single-Column and Two-Column Protocol Comparison)

### Experimental Setup

[0569] As outlined above in the Purification Protocol Flowchart, two alternative purification approaches are feasible depending mainly on individual preferences regarding ethanol precipitation and procedure time. In the following experiments, which are illustrated in Figures 19A-19C, lacZ siRNA was purified according to the single-column and two-column protocols described earlier. The siRNA obtained was tested for specificity and functionality in transfection experiments using GripTite™ 293 MSR cells, which contained either luciferase or  $\beta$ -galactosidase gene constructs in reporter plasmids, as described in detail above. In the case of the single-column purification protocol, elution was performed with Elution Buffer containing ethanol at final concentrations of between 5 and 30%. Transfection experiments were performed in duplicate.

### Results and Discussion

[0570] Figure 19A shows gel analysis results of crude lacZ siRNA, siRNA purified using the two-column protocol, various fractions of the single-column purification protocol, as well as chemically synthesized siRNA analyzed on a 4% E-Gel, which were used for functional testing. Green Fluorescent Protein (GFP) siRNA, by virtue of being chemically synthesized, does not contain any long dsRNA impurities. The siRNA that was purified with the two-column protocol and siRNA fractions eluted with 20, 25, and 30% EtOH using the single-column protocol appear to be devoid of intermediate Dicer reaction products and full-length dsRNA template. Therefore, these siRNA preparations are expected to be potent and specific in the suppression of their target genes and are not expected to exhibit any of the adverse effects associated with the presence of long dsRNA. Unpurified lacZ siRNA contains significant amounts of undigested and partially digested long dsRNA

molecules and is hence expected to result in cell death upon transfection. Likewise, siRNA purified with the single-column protocol and eluted with Elution Buffer containing 5, 15, and 20% EtOH is expected to result in cell death, albeit at decreasing degrees as ethanol concentration increases.

[0571] Figure 19 A:

Lane 1: 10 bp DNA Ladder (Invitrogen Cat #18021-015) - The 10-bp fragment only shows as a faint band.

Lane 2: Chemically synthesized, unpurified Green Fluorescent Protein (GFP) siRNA

Lane 3: Crude lacZ siRNA reaction mixture

Lane 4: LacZ siRNA purified using the two-column protocol (see flowchart above)

Lane 5: LacZ siRNA eluted with 5% EtOH-containing Elution Buffer according to the single-column protocol

Lane 6: LacZ siRNA eluted with 10% EtOH-containing Elution Buffer according to the single-column protocol

Lane 7: LacZ siRNA eluted with 15% EtOH-containing Elution Buffer according to the single-column protocol

Lane 8: LacZ siRNA eluted with 20% EtOH-containing Elution Buffer according to the single-column protocol

Lane 9: LacZ siRNA eluted with 25% EtOH-containing Elution Buffer according to the single-column protocol

Lane 10: LacZ siRNA eluted with 30% EtOH-containing Elution Buffer according to the single-column protocol

[0572] The effects of lacZ siRNA on luciferase activity are shown in Figure 19B. This is a negative control experiment. Since lacZ siRNA does not have sequence homology to the luciferase gene, its activity should remain unperturbed by the presence of lacZ siRNA. Any changes in luciferase activity will thus be attributed to nonspecific effects such as the effect that the presence of long dsRNA may have on the transfected cells or the effects of transfection itself. Cells that do not carry the reporter plasmid for luciferase

(untransfected) do not exhibit luciferase activity. Cells transfected with the reporter plasmid for luciferase (reporters alone) exhibit baseline luciferase activity serving as a point of reference for the action of siRNA in the following experiments. Transfection of cells carrying luciferase reporter plasmid with chemically synthesized, unrelated GFP siRNA (GFP siRNA) did not alter luciferase activity, as expected. Unpurified, crude lacZ Dicer reaction containing undigested and partially digested long dsRNA resulted in cell death and a concomitant lack of luciferase activity (lacZ dicing reaction). Transfection of target cells with lacZ siRNA purified using the two-column purification protocol (lacZ d-siRNA) did not suppress luciferase activity as expected. However, nonspecific induction of luciferase activity by about 40% was apparent. LacZ siRNA obtained by elution with Elution Buffer containing 5, 10, or 15% ethanol using the single-column protocol (lacZ fract 5, lacZ fract 10, lacZ fract 15) resulted in suppression of luciferase activity. This observation, however, is attributed to residual long dsRNA template and partial digestion products thereof in these fractions eliciting cell death as observed for unpurified Dicer reactions. The observed suppression of luciferase activity in these cases is not the result of specific siRNA action. LacZ siRNA obtained by elution with Elution Buffer containing 20, 25, and 30% EtOH did not alter luciferase activity. Hence, these fractions of purified siRNA do not elicit nonspecific effects such as induction or suppression of luciferase activity upon transfection.

[0573] The effects of lacZ siRNA on its target transcripts, as evidenced and measurable through the activity of  $\beta$ -galactosidase, are shown in Figure 19C. Cells that do not carry the reporter plasmid for  $\beta$ -galactosidase (untransfected) do not exhibit  $\beta$ -galactosidase activity. Cells transfected with the reporter plasmid for  $\beta$ -galactosidase (reporters alone) exhibit baseline  $\beta$ -galactosidase activity serving as a point of reference for the action of siRNA in the following experiments. Transfection of cells carrying  $\beta$ -galactosidase reporter plasmid with chemically synthesized, unrelated GFP siRNA (GFP siRNA) did not alter  $\beta$ -galactosidase activity. Unpurified, crude lacZ Dicer reaction

containing undigested and partially digested long dsRNA resulted in cell death and a concomitant lack of  $\beta$ -galactosidase activity (lacZ dicing reaction). Transfection of target cells with lacZ siRNA purified using the two-column purification protocol (lacZ d-siRNA) suppressed  $\beta$ -galactosidase activity by approximately 70%. LacZ siRNA obtained by elution with Elution Buffer containing 5, 10, or 15% ethanol using the single-column protocol (lacZ fract 5, lacZ fract 10, lacZ fract 15) resulted in suppression of  $\beta$ -galactosidase activity. This observation, however, may be attributed to residual long dsRNA template and partial digestion products thereof in these fractions, eliciting cell death as observed for unpurified Dicer reactions. In addition,  $\beta$ -galactosidase activity in surviving cells may further be suppressed by the presence of siRNA specific for the lacZ gene. Thus, while suppression appears to be efficient, it is mainly caused by cell death and not by the specific action of the siRNA used. LacZ siRNA obtained by elution with Elution Buffer containing 20, 25, and 30% EtOH did profoundly suppress the activity of the  $\beta$ -galactosidase enzyme by approximately 80%. In the latter case cells appeared healthy after transfection with purified siRNA. Hence, these fractions of purified siRNA are highly effective and specific in the suppression of their targeted mRNA.

[0574] Fractionated siRNA samples used were obtained using either the single-column or two-column protocol as a means of purification. The effects of lacZ siRNA are specific for the  $\beta$ -galactosidase gene due to sequence homologies and a reduction of  $\beta$ -galactosidase activity is expected as a result of the presence of lacZ siRNA.

[0575] Figures 19 B and C:

Untransfected: Cells have not been transfected with reporter plasmids carrying the luciferase or  $\beta$ -galactosidase gene

Reporters alone: Cells have been transfected with reporter plasmid only, but not with siRNA

GFP siRNA: Transfection with chemically synthesized, crude siRNA specific for the green fluorescent protein gene



LacZ dicing reaction: Transfection with crude, unpurified lacZ siRNA from Dicer reaction

LacZ d-siRNA: Transfection with lacZ siRNA purified using the two-column protocol

LacZ frac 5: Transfection with lacZ siRNA from 5% EtOH containing fraction (single-column protocol)

LacZ frac 10: Transfection with lacZ siRNA from 10% EtOH containing fraction (single-column protocol)

LacZ frac 15: Transfection with lacZ siRNA from 15% EtOH containing fraction (single-column protocol)

LacZ frac 20: Transfection with lacZ siRNA from 20% EtOH containing fraction (single-column protocol)

LacZ frac 25: Transfection with lacZ siRNA from 25% EtOH containing fraction (single-column protocol)

LacZ frac 30: Transfection with lacZ siRNA from 30% EtOH containing fraction (single-column protocol)

Purification of siRNA Generated by Dicer or RNase III

#### Experimental Setup

[0576] One-kb dsRNA transcript of either lacZ or luciferase was incubated with Dicer or RNaseIII to generate double-stranded siRNA products. Dicer reactions were carried out using a protocol as described in the BLOCK-iT™ Complete Dicer RNAi Kit (Invitrogen cat. # K3650-01). Digestion with RNaseIII (Ambion, cat. # 2290) was performed according to the manufacturer's suggestions. Crude RNase III and Dicer siRNA reactions were purified using the single-column and two-column purification protocol and subsequently tested for functionality.

## Results and Discussion

[0577] The Dicer enzyme is a member of the RNaseIII family of ribonucleases and digests long dsRNA templates into 21-23 nucleotide, double-stranded siRNA that have been shown to function as key intermediates in triggering sequence specific RNA degradation during posttranscriptional gene silencing. Likewise, RNaseIII digests long dsRNA templates into short double-stranded siRNA molecules. However, the siRNA generated by RNaseIII is generally only approximately 12 – 15 base pairs long. Dicer enzyme is found in all eukaryotic cells and RNaseIII is mainly found in prokaryotes. Dicer enzyme is speculated to bind to the ends of long dsRNA and progressively cleave the template dsRNA. The mode of action of RNaseIII may involve random cleavage of template dsRNA into smaller, compared to the Dicer enzyme, 12-15 bp siRNA fragments. The RNaseIII enzyme is considerably more active than the corresponding Dicer enzyme, which leads to complete digestion of template dsRNA by RNaseIII enzyme, while Dicer enzyme, even after prolonged digestion, results in only incomplete digestion of template dsRNA. These findings are illustrated in Figure 20A. Neither enzyme requires ATP for function. However, both enzymes require divalent metal cations and a specific, optimal pH range for optimal activity, which are provided by enzyme-specific reaction buffers. The purification procedures for siRNA used here result in the removal of proteins and buffer components. The purified siRNA is resuspended in RNase-free water in the final purification step independent of the purification protocol used. Short interfering RNA generated by the action of RNase III as well as by Dicer enzyme were successfully purified using Invitrogen's spin columns applying either the single- or two-column purification protocol as shown in Figure 20B.

[0578] Figures 20A and 20B show purification of siRNA generated with Dicer and RNaseIII

[0579] Figure 20A:

Lane 2: unpurified lacZ siRNA cleaved by RNaseIII,

Lane 3 unpurified luciferase siRNA cleaved by RNaseIII,

Lane 4: unpurified lacZ siRNA cleaved by Dicer,

Lane 5: unpurified luciferase siRNA cleaved by Dicer

[0580] Figure 20B:

Lane 1, 5,9: lacZ siRNA cleaved by RNaseIII,

Lane 2, 6,10: luciferase siRNA cleaved by RNaseIII,

Lane 3, 7,11 lacZ siRNA cleaved by Dicer,

Lane 4, 8,12 luciferase siRNA cleaved by Dicer

#### Conclusion

[0581] Short interfering RNA generated by digestion of long dsRNA templates with either Dicer or RNaseIII enzyme can be efficiently purified using the single-column or two-column purification protocol.

#### Functional Testing of SiRNA Preparations with FlpIn 293 luc Cells

#### Experimental Setup

[0582] Short interfering RNA was generated by digestion of long dsRNA templates (1µg) with either RNase III or Dicer enzyme. Samples were purified using the single- and two-column purification protocol. Concentrations of purified siRNA were determined by A260 measurements and 20 ng of purified sample was used for each transfection into FlpIn 293 luc cells.

#### Results and Discussion

[0583] Transfection experiments were performed in 5 experimental groups with each experiment being conducted in duplicate. Experimental group 1 consisted of two control reactions of mock transfected (Mock), i.e. transfection with transfection agent but without siRNA, as well as FlpIn 293 luc cells expressing baseline levels of luciferase (Untransfected). The luciferase activity was measured in these latter two experiments and served as

reference for luciferase activity that was determined in group 2–5 experiments. In experimental groups 2 and 3 the effect of luciferase activities by Dicer generated luciferase specific siRNA (luc siRNA) and  $\beta$ -galactosidase specific siRNA (lacZ siRNA) were assessed. Likewise, in groups 4 and 5 the effect of siRNA generated with RNaseIII enzyme on luciferase activity was assessed.

[0584] siRNA (20 ng luc Dicer reaction) resulted in cell death and nonspecific lack of luciferase activity (see Figure 21). SiRNA that was purified using the single-column purification protocol, where siRNA was eluted with Elution Buffer containing 25 or 30% EtOH (20 ng luc Dicer 25% pur. & 20 ng luc Dicer 30% pur.), resulted in efficient suppression of luciferase activity by more than 80%. Equally efficient suppression was achieved with siRNA purified using the two-column purification protocol (20 ng luc Dicer 2 col. pur.) and with the latter siRNA that was subjected to an additional step of EtOH precipitation (20 ng luc d-siRNA (EtOH)). Thus, luciferase specific siRNA purified with either the single-column or two-column purification protocol is highly potent in suppressing the activity of luciferase.

[0585] As shown in experimental group 3 in Figure 21, all purified and  $\beta$ -galactosidase-specific siRNA samples generated with the Dicer enzyme failed, as expected, to significantly change expression levels of the luciferase gene as determined by assessing luciferase activity. Variations in the activity of the luciferase enzyme caused by  $\beta$ -galactosidase-specific siRNA were generally less than 10%. As observed earlier, unpurified Dicer-generated siRNA (20 ng luc Dicer reaction) resulted in cell death and nonspecific lack of luciferase activity. Thus,  $\beta$ -galactosidase-specific siRNA purified with the single-column or two-column protocol does not cause any significant nonspecific induction or suppression of bystander proteins, in this case luciferase.

[0586] In experimental groups 4 and 5 the effect of luciferase specific siRNA (luc siRNA) as well as  $\beta$ -galactosidase enzyme specific siRNA (lacZ siRNA) generated with RNaseIII enzyme (Ambion) on luciferase activity was assessed. Unlike unpurified Dicer reactions, which contain significant amounts of undigested or partially digested long dsRNA template, unpurified

RNaseIII reactions do not contain significant amounts of undigested or partially digested long dsRNA template as previously shown in Figure 20A. Consequently, transfection with unpurified RNaseIII reaction products (20 ng luc RNaseIII reaction & 20 ng lacZ RNaseIII reaction) does not lead to cell death and concomitant nonspecific reduction of luciferase activity. RNase III digestion products are in the 13 – 15 bp size range, which is well below the size range reported for potent siRNA (~20-23bp). Consequently, purified RNaseIII-generated luciferase specific siRNA (20 ng luc RNaseIII 25% pur., 20 ng luc RNaseIII 30% pur, and 20 ng luc RNaseIII 2 col. pur.) suppressed luciferase activity by only approximately 25% under the conditions used here. This lack of efficient suppression at the siRNA concentrations used may be attributable to a lack of functional siRNA present after digestion with RNaseIII since the siRNA size generated by RNaseIII is less than 20 base pairs (see Figures 20A and 20B). Czauderna et al.(Nucleic Acids Research 2003) reported that synthetic siRNAs shorter than 19 base pairs in size were not effective in suppressing gene expression. Concentrations of up to 200 ng/transfection of RNaseIII-generated siRNA were tested. However, even at these elevated amounts no significant suppression was observed. As shown in experimental group 5, neither unpurified nor purified lacZ siRNA that was generated by digestion of long dsRNA templates with RNaseIII had any effect on luciferase activity.

#### Functional Testing of siRNA Preparations with GripTite™ MSR Cells

##### Experimental setup

[0587] Two reporter plasmids (see above) expressing luciferase and  $\beta$ -galactosidase, respectively, were co-transfected into GripTite™ 293 MSR cells with siRNA specific for luciferase mRNA (luc) or  $\beta$ -galactosidase mRNA (lacZ) generated by Dicer or RNaseIII using the experimental scheme described in the previous experiment. Luciferase and  $\beta$ -galactosidase activity

was determined as described above to assess the effect of specific siRNA preparations on the expression of the two gene transcripts under investigation.

## Results and Discussion

[0588] Results presented in Figure 22A demonstrate the effect of different luc siRNA and lacZ siRNA preparations generated with Dicer and RNaseIII enzyme on  $\beta$ -galactosidase activity. The results obtained are in good agreement with the results shown in Figure 21. In brief, Griptite 293 MSR cells transfected with the reporter plasmid alone (Reporters Only) exhibited reference levels of  $\beta$ -galactosidase activity. Cells not transfected with the reporter plasmid (Mock) did not yield any  $\beta$ -galactosidase activity. As seen previously, crude Dicer reactions (20 ng luc Dicer reaction & 20 ng lacZ Dicer reaction) caused cell death and nonspecific suppression of  $\beta$ -galactosidase activity, while this effect was not observed with crude RNaseIII reactions (20 ng luc RNaseIII reaction & 20 ng lacZ RNaseIII reaction). All preparations of purified, Dicer-generated lacZ siRNA efficiently suppressed expression of  $\beta$ -galactosidase activity by more than 80%. On the other hand, neither preparation of the negative control luc siRNA affected  $\beta$ -galactosidase activity to any significant degree. SiRNA generated by digestion with RNaseIII elicited similar responses to those observed above. Suppression of  $\beta$ -galactosidase activity by lacZ siRNA preparations was inefficient with maximum suppressions of 40%. However, from the results shown in Figure 22A it is apparent that luciferase specific siRNA preparations generated with RNaseIII enzyme caused significant nonspecific induction of the  $\beta$ -galactosidase gene.

[0589] Results presented in Figure 22B demonstrate the effect of different luc siRNA and lacZ siRNA preparations generated with Dicer and RNaseIII enzyme on luciferase activity. The results obtained are in good agreement with the results shown in Figure 21. In brief, Griptite 293 MSR cells transfected with the reporter plasmid alone (Reporters Only) exhibited

reference levels of luciferase activity. Cells not transfected with the reporter plasmid (Mock) did not yield any luciferase activity. Crude Dicer reactions (20 ng luc Dicer reaction & 20 ng lacZ Dicer reaction) caused cell death and nonspecific suppression of luciferase activity, while this effect was not observed with crude RNaseIII reactions (20 ng luc RNaseIII reaction & 20 ng lacZ RNaseIII reaction). All preparations of purified, Dicer-generated luc siRNA efficiently suppressed expression of luciferase activity by more than 90%. On the other hand, neither preparation of the negative control lacZ siRNA suppressed luciferase activity. However, in the series of experiments shown here, luciferase activity was stimulated by up to 40% by  $\beta$ -galactosidase specific lacZ siRNA. SiRNA generated by digestion with RNaseIII elicited similar responses to those observed above. The suppression of luciferase activity by luc siRNA preparations was inefficient with maximum suppressions of approximately 20%. The effects of lacZ siRNA preparations generated with RNaseIII on luciferase activity were inconsistent with both induction (20 ng lacZ RNaseIII 25% pur.) and suppression (20 ng lacZ RNaseIII 30% pur. & 20 ng lacZ RNaseIII 2 col. pur.) being observed.

#### Conclusion

[0590] SiRNA generated by digestion of long dsRNA templates with Dicer enzyme and purified using either the single-column or two-column purification protocol efficiently suppressed gene specific expression with minimal nonspecific induction of bystander proteins. Short interfering RNA generated by digestion of long dsRNA templates with RNaseIII enzyme, while efficiently purified with either the single-column or two-column purification protocol, did not perform well under the experimental conditions used here.

## Column Capacity and Recovery Determination

### Experimental setup.

[0591] These experiments were intended to determine the recovery of RNA, tRNA and a 1-kb dsRNA fragment, after binding to the glass fiber matrix of the spin column as a function of elution volume. The experiments were also designed to provide information about the general loading capacity of the spin column for short double-stranded nucleic acids and long dsRNA fragments, the latter are used as templates for RNase digestion assays. In order to assess the column capacity for siRNA, yeast tRNA was used, because it was available in the quantities needed. Yeast tRNA constitutes a sensible alternative for column testing to siRNA as its linear, single-stranded size is approximately 75 nucleotides that are involved in extensive secondary structure formation, i.e. tRNA is present predominantly in dsRNA form. The tRNA used here migrates like a 40-bp double-stranded nucleic acid fragment on agarose gels. The efficiency of recovery and approximate loading capacity was also determined for a 1-kb dsRNA fragment. These long dsRNA fragments serve as templates for Dicer and RNaseIII digestion and require purification after clean up of the transcription reaction with DNaseI and RNaseA and prior to Dicer/RNaseIII digestion for generating siRNA. Purifications were carried out using the single-column purification protocol with 10 – 1000 µg of yeast tRNA or 4 – 240 µg of the 1-kb dsRNA fragment. Bound dsRNA was eluted from the spin columns with either a single elution of 100 µl DEPC-treated water or two successive elutions of 50 µl DEPC-treated water. Amounts of eluted RNA were quantified by A260 measurements and compared to the initial amount of RNA loaded.

### Results and Discussion

[0592] Ten µg of tRNA were eluted with either a single 100-µl elution or two 50-µl elutions with an efficiency exceeding 90% (Figure 23A). Amounts of



tRNA of up to 1 mg can be eluted with efficiencies of approximately 80%, independent of whether a single 100- $\mu$ l elution or two 50- $\mu$ l elutions were used. Recovery can be further increased to about 95% with a second 100- $\mu$ l elution or a third 50- $\mu$ l elution. It shall be noted that for yeast tRNA amounts in excess of about 100  $\mu$ g the addition of Binding Buffer and EtOH to the sample results in precipitation of presumably tRNA. The results shown in Figure 23A demonstrate that tRNA, and by correlation siRNA, can be recovered almost quantitatively from the spin column matrix by elution with DEPC-treated water. Also, the results show that the column capacity exceeds 1 mg for tRNA/siRNA.

[0593] Figure 23B shows the recovery results obtained with a 1-kb dsRNA fragment loaded at amounts ranging from 4 – 240  $\mu$ g. Independent of whether a single 100- $\mu$ l elution or two 50- $\mu$ l elutions were used recovery efficiency was about 90%. It shall be noted that the long dsRNA fragment was more susceptible to form a precipitate after the addition of Binding Buffer and EtOH than tRNA. Loading of dsRNA amounts exceeding about 500  $\mu$ g resulted in progressively decreasing recoveries with either a single 100- $\mu$ l elution or two 50- $\mu$ l elutions, which could be improved with additional elutions.

#### Conclusion

[0594] Short dsRNA, e.g., siRNA or tRNA, as well as long dsRNA fragments can be efficiently eluted after binding to the spin column with DEPC-treated water. No major differences in recovery were observed for either a single elution with 100  $\mu$ l or two successive 50- $\mu$ l elutions.

#### Clean-up of Long dsRNA Substrate and tRNA

#### Experimental Setup

[0595] Three different sizes of long dsRNA (100, 500 and 1 kb) of the lacZ gene were generated by T7 polymerase reactions as described above. The

100-bp and 500-bp lacZ dsRNA fragments were generated using primer 1 (see above) and primer 9 (5'- GCA TCG TAA CCG TGC ATC 3' (SEQ ID NO: 13) and primer 10 (5' GCG AGT GCC AAC ATG G 3' (SEQ ID NO: 14), respectively, for the first round PCR. Primer 3 (see above) in combination with primer 11 (5'-GAC TCG TAA TAC GAC TCA CTA TAG GTA CTG CAT CG T AAC CGT GCA TC -3' (SEQ ID NO: 15)) and primer 12 (5'-GAC TCG TAA TAC GAC TCA CTA TAG GTA CTG CGA GTG GCA ACA TGG-3' (SEQ ID NO: 16)), respectively, were used for the second round of PCR. The 1-kb dsRNA fragment was generated as described above. All dsRNA fragments generated were cleaned up by DNase I and RNase A digestion to remove DNA and single-stranded RNA from the reactions before purification using a modified single-column protocol (see below).

## Results and Discussion

[0596] Long dsRNA intended for Dicer or RNaseIII digestion has to be cleaned up with DNase I and RNase A to remove DNA and unhybridized single-stranded RNA. Subsequently, the latter enzymes, their digestion products, and buffer components need to be removed prior to digestion of the long dsRNA templates with Dicer or RNaseIII. A modified version of the single-column protocol was used to purify long dsRNA suitable for Dicer and RNaseIII digestion. Binding capacity and recovery of dsRNA from the glass fiber filters was determined previously. Purification results of long dsRNA and tRNA are shown in Figures 24A and 24B.

[0597] Purification of dsRNA (50ul sample)

1. Add 150 µl of Binding Buffer and mix well
2. Add 600 µl of 100% EtOH (Final EtOH concentration of 75%)
3. Mix well and load onto column
4. Centrifuge at 14000 rpm for 1 min
5. Wash with 500 µl of diluted Wash Buffer
6. Repeat the washing step
7. Centrifuge at 14000 rpm for 1 min to dry column

8. Add 100 µl of DEPC-treated water
9. Wait for 1 min
10. Centrifuge at 14000 rpm for 1 min to recover dsRNA

[0598] Figure 24A:

Lane 1: 1 kb Plus DNA Ladder (Invitrogen)

Lane 3; 100-bp lacZ dsRNA fragment

Lane 5: 500-bp lacZ dsRNA fragment

Lane 7: 1-kb lacZ dsRNA fragment

[0599] Figure 24 B:

Lane 1: 10 bp DNA Ladder (Invitrogen)

Lane 3: Unpurified yeast tRNA (0.3 µg)

Lane 4: Cleaned-up yeast tRNA (0.3 µg))

Lane 6: Unpurified (1.5 µg)

Lane 7: Cleaned-up yeast tRNA (1.5 µg))

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- [0608] The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations, which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms “comprising,” “consisting essentially of,” and “consisting of” may be replaced with either of the other two terms. The terms and expressions

that have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed herein, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

[0609] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein. Other aspects of the invention are within the following claims.

[0610] All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

WHAT IS CLAIMED IS:

1. A method for preparing nucleic acids of a preselected size comprising:
  - (a) adding a fluid mixture or, in any order, fluids or combinations of fluids that contain the components of said fluid mixture to a sample comprising nucleic acids of said preselected size and other nucleic acids, to produce a binding mixture;
  - (b) filtering said binding mixture through an affinity column, wherein nucleic acids of said preselected size and said other nucleic acids bind to a composition within the affinity column; and optionally washing the bound nucleic acids; and
  - (c) eluting the bound nucleic acids of said preselected size with a second fluid mixture, to produce an eluate,wherein nucleic acids of said preselected size are present in said eluate, and said other nucleic acids remain bound to the composition within said affinity column.
2. A method for preparing nucleic acids of a preselected size comprising:
  - (a) adding a fluid mixture or, in any order, fluids or combinations of fluids that contain the components of said fluid mixture to a sample comprising nucleic acids of a preselected size and other nucleic acids having a size other than said preselected size to produce a binding mixture;
  - (b) filtering said binding mixture through an affinity column and collecting the flow-through therefrom,wherein said nucleic acids of a preselected size are present in said flow-through, and said other nucleic acids remain bound to the composition within said affinity column.
3. A method for preparing nucleic acids of a preselected size comprising:
  - (a) adding a first fluid mixture or, in any order, fluids or combinations of fluids that contain the components of said first

fluid mixture, to a sample comprising nucleic acids of a preselected size and other nucleic acids having a size other than said preselected size to produce a first binding mixture;

- (b) filtering said first binding mixture through a first affinity column, wherein nucleic acids of said preselected size pass through a composition within the first affinity column, and said other nucleic acids are retained, to produce a first flow-through solution;
- (c) adding a second fluid mixture or, in any order, fluids or combinations of fluids that contain the components of said second fluid mixture to said first flow-through solution, to produce a second binding mixture;
- (d) filtering the second binding mixture through a second affinity column, wherein nucleic acids of said preselected size bind to a composition within the second affinity column and, optionally, washing the bound nucleic acids; and
- (e) eluting nucleic acids of said preselected size with a third fluid mixture, to produce an eluate,

wherein nucleic acids of said preselected size molecules are present in said eluate.

4. A method for preparing nucleic acids of a preselected size comprising:

- (a) adding a first fluid mixture or, in any order, fluids or combinations of fluids that contain the components of said first fluid mixture, to a nuclease reaction mix, to produce a first binding mixture, wherein the first fluid mixture is essentially free of alcohol;
- (b) filtering said first binding mixture through a first affinity column, wherein longer RNA molecules in the first binding mixture (*i.e.*, RNA having a length of about "y" bp to about "z" or more bp) bind to, and shorter RNA molecules (*i.e.*, RNA

having a length of about 5 bp to about "y" bp) pass through, a composition within said first affinity column, to produce a first flow-through solution comprising RNA having a length of about 5 bp to about "y" bp;

- (c) eluting said longer RNA from said first affinity column, to produce a first eluate comprising longer RNA molecules having lengths in the range of about "y" bp to about "z" or more bp;
- (d) adding one or more alcohol(s) to said first flow-through solution to bring the final concentration to about 10% (v/v), to produce a second binding mixture;
- (e) filtering said second binding mixture through a second affinity column, wherein longer RNA molecules in said second binding mixture (*i.e.*, RNA having a length of about "x" bp to about "y" or more bp) bind to, and shorter RNA molecules (*i.e.*, RNA having a length of about 5 bp to about "x" bp) pass through, a composition within said second affinity column, to produce a second flow-through solution comprising RNA having a length of about 5 bp to about "x" bp;
- (f) eluting the longer RNA from said second affinity column, to produce a second eluate comprising longer RNA molecules having lengths in the range of about "x" bp to about "y" bp;
- (g) adding one or more alcohol(s) to said second flow-through solution to bring the final concentration to about 10% (v/v) more than the first flow-through solution, to produce a third binding mixture;
- (h) filtering said third binding mixture through a third affinity column, wherein longer RNA molecules in said second binding mixture (*i.e.*, RNA having a length of about "w" bp to about "x" bp) bind to, and shorter RNA molecules (*i.e.*, RNA having a length of about 5 bp to about "w" bp) pass through a composition within said third affinity column, to produce a

third flow-through solution comprising RNA having a length of about 5 bp to about “w” bp; and

- (i) eluting the longer RNA from said second affinity column, to produce a third eluate comprising longer RNA molecules having lengths in the range of about “w” bp to about “x” bp;

wherein “w” < “x” < “y” < “z”, and wherein said first eluate comprises RNA molecules having an average length greater than that of the RNA molecules in said second eluate, and said second eluate comprises RNA molecules having an average length greater than that of said RNA molecules in said third eluate.

- 5. The method of any one of claims 1-4, wherein at least one of said affinity columns comprises glass fibers.
- 6. The method of any one of claims 1-4, wherein said nucleic acids of a preselected size are selected from the group consisting of DNA, PNA and RNA molecules.
- 7. The method of any one of claims 1-4, wherein said nucleic acids of a preselected size are RNA molecules.
- 8. The method of any one of claims 1-4, wherein said RNA molecules are double-stranded.
- 9. The method of claim 7, wherein said RNA molecules are micro-RNA molecules.
- 10. The method of claim 7, wherein said RNA molecules are RNAi molecules.



11. The method of claim 10, wherein said RNAi molecules are selected from the group consisting of shRNA, stRNA, siRNA, e-siRNA, and d-siRNA.
12. The method of any one of claims 1-4, wherein said preselected size ranges from about 15 to about 1,000 nucleotides or base pairs in length.
13. The method of any one of claims 1-4, wherein said preselected size ranges from about 15 to about 100 nucleotides or base pairs in length.
14. The method of any one of claims 1-4, wherein said preselected size ranges from about 18 to about 50 nucleotides or base pairs in length.
15. The method of any one of claims 1-4, wherein said preselected size ranges from about 20 to about 30 nucleotides or base pairs in length.
16. The method of any one of claims 1-4, wherein said preselected size ranges from about 20 to about 25 nucleotides or base pairs in length.
17. The method of any one of claims 1-4, wherein one or more of said fluid mixtures comprises one or more alcohols.
18. The method of claim 17, wherein said alcohol is selected from the group consisting of methanol, ethanol, propanol, isopropanol, butanol, isobutyl alcohol, tertiary butyl alcohol, 1-hexanol, 2-hexanol, and 3-hexanol.
19. The method of claim 17, wherein at least one of said fluid mixtures comprises about m% (v/v) of said alcohol, wherein m is any whole integer from 1 to 50.

20. The method of claim 17, wherein at least one of said fluid mixtures comprises about  $n\%$  (v/v) of said alcohol, wherein  $n$  is any whole integer from 50 to 100.
21. The method of claim 17, wherein at least one of said fluid mixtures is an azeotrope of water and one or more alcohols.
22. The method of any one of claims 1-4, wherein one or more of said binding mixtures comprises one or more alcohols.
23. The method of claim 22, wherein said alcohol is selected from the group consisting of methanol, ethanol, propanol, isopropanol, butanol, isobutyl alcohol, tertiary butyl alcohol, 1-hexanol, 2-hexanol, and 3-hexanol.
24. The method of claim 22, wherein at least one of said binding mixtures comprises about  $m\%$  (v/v) of said alcohol, wherein  $m$  is any whole integer from 1 to 50.
25. The method of claim 22, wherein at least one of said binding mixtures comprises about  $n\%$  (v/v) of said alcohol, wherein  $n$  is any whole integer from 50 to 100.
26. The method of claim 22, wherein at least one of said binding mixtures is an azeotrope of water and one or more alcohols.
27. The method of any one of claims 1-4, wherein at least one of said binding solutions, or at least one of said fluid mixtures, is a buffer or a weak buffer.

28. The method of any one of claims 1-4, wherein said nucleic acids of a preselected size and said other nucleic acids are both RNA molecules.
29. The method of claim 28, wherein both of said RNA molecules are double-stranded RNA molecules.
30. The method of claim 28, wherein said other nucleic acids are substrate RNA molecules for an enzyme, and said nucleic acids of a preselected size are the products of a reaction catalyzed by said enzyme.
31. The method of claim 30, wherein said enzyme is a ribonuclease.
32. The method of claim 31, wherein said ribonuclease is a member of the RNase III family of ribonucleases.
33. The method of claim 32, wherein said ribonuclease is selected from the group consisting of a DICER, an RNase III, an RNase A, RNase T1 and nuclease S1.
34. The method of claim 10, wherein said DICER is recombinant.
35. The method of claim 30, wherein said substrate RNA is prepared by a process selected from the group consisting of chemical synthesis and enzymatic synthesis.
36. The method of claim 30, wherein said substrate RNA is prepared by *in vitro* transcription.
37. The method of any of claims 1-4, wherein said sample is from a biological system.

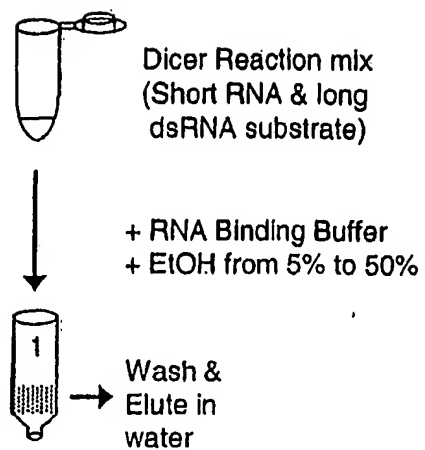
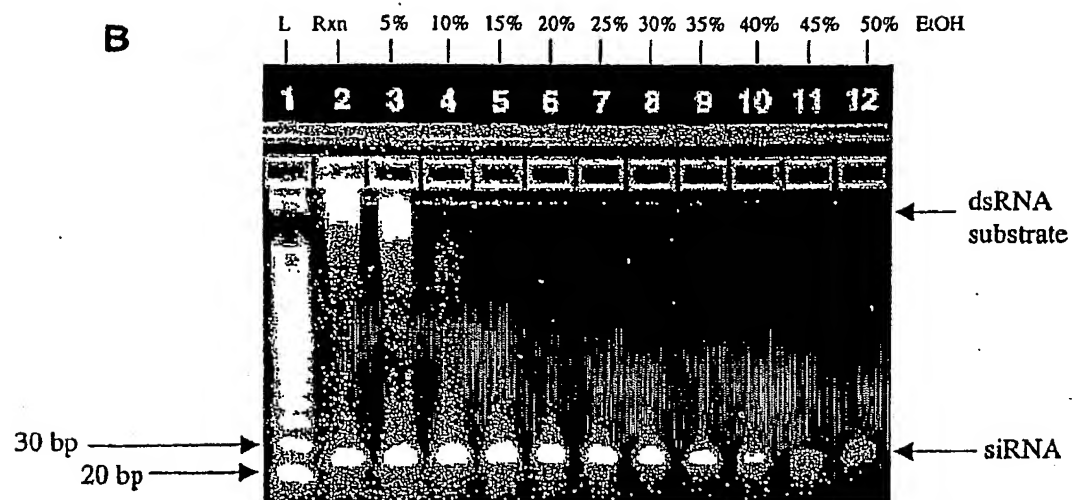
38. The method of claim 3, further comprising drying said second affinity column between (d) and (e).
39. The method of claim 3, wherein said first binding solution comprises about m% (v/v) of said alcohol, wherein m is any whole integer from 1 to 50.
40. The method of claim 3, wherein said first binding solution comprises from about 30% to about 45% (v/v) of said alcohol.
41. The method of claim 3, wherein said second binding solution comprises about n% (v/v) of said alcohol, wherein n is any whole integer from 50 to 100.
42. The method of claim 3, wherein said second binding solution comprises from about 65% to about 75% (v/v) of said alcohol.
43. The method of claim 3, wherein at least one of said binding solutions, or at least one of said fluid mixtures, is an azeotrope of water and one or more alcohols.
44. The method of claim 3, wherein at least one of said binding solutions, or at least one of said fluid mixtures, is a buffer or a weak buffer.
45. The method of claim 3, wherein said first affinity column and said second affinity column have the same composition.
46. The method of claim 1, wherein nucleic acids of a preselected size prepared according to the method have one or more desirable properties selected from the group consisting of enhanced activity,

enhanced specificity, enhanced specific activity, reduced toxicity, enhanced *in vivo* stability, and enhanced *in vitro* stability.

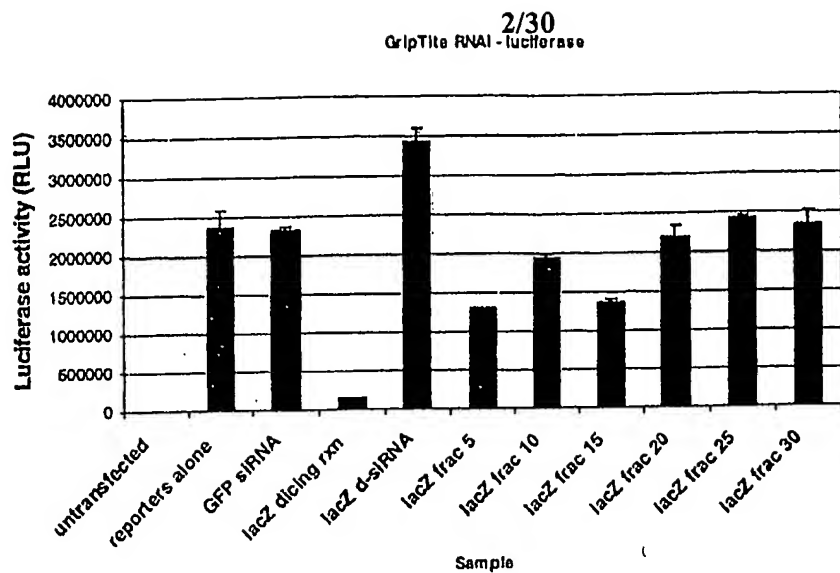
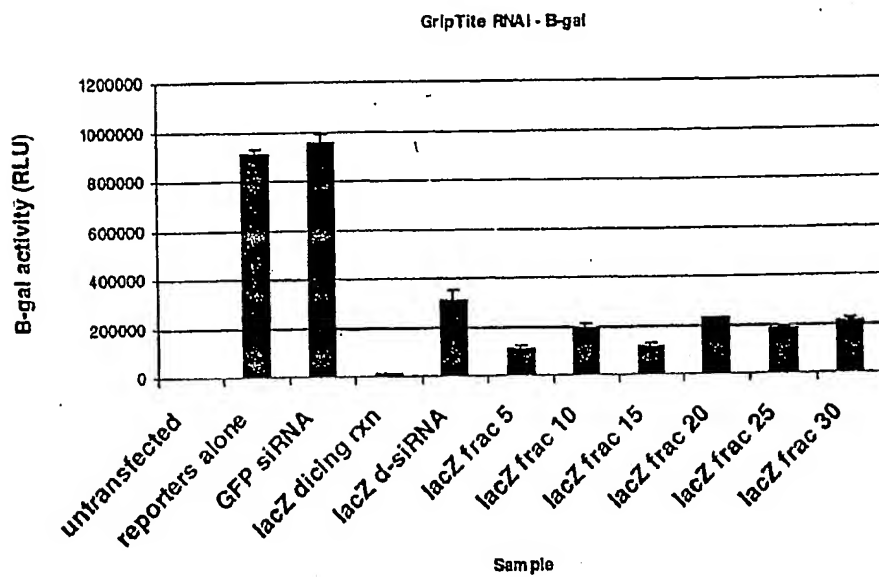
47. A kit comprising at least one component selected from the group consisting of an affinity column, a buffer, an alcoholic solution, an enzyme, a nucleic acid, a transfection agent, and one more sets of instructions.
48. The kit of claim 47, wherein said enzyme is a ribonuclease.
49. The kit of claim 48, wherein said ribonuclease is selected from the group consisting of DICER and RNase III.
50. The kit of claim 47, wherein said kit comprises one or more primers.
51. The kit of claim 47, wherein said nucleic acid is a vector for transcribing double-stranded RNA that is a substrate for said enzyme.
52. The kit of claim 51, further comprising primers for directing transcription of said vector.
53. The kit of claim 51, further comprising an RNA polymerase that catalyzes transcription of said vector.
54. The kit of claim 53, wherein said RNA polymerase is selected from the group consisting of T3 RNA polymerase, T7 RNA polymerase, and SP6 RNA polymerase.
55. The kit of claim 48, wherein said set of instructions comprises instructions for optimizing ribonuclease reactions.

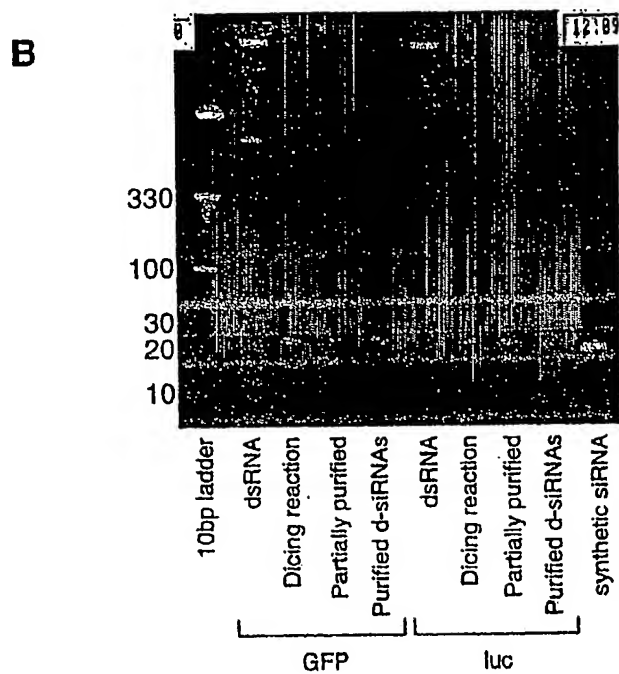
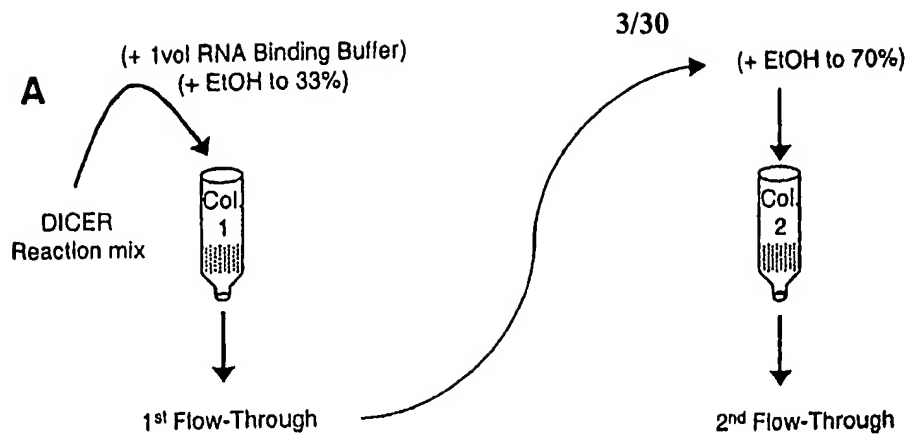
56. The kit of claim 48, wherein said kit comprises a buffer that supports RNase reactions, or which can be diluted to generate a buffer that supports RNase reactions.
57. The kit of claim 48, wherein said kit comprises a solution for stopping RNase reactions.
58. The kit of claim 47, wherein said affinity column comprises glass fibers.
59. A method of identifying Short RNA molecules that modulate the expression of one or more genes in a biological system, comprising:
  - (a) preparing a plurality of candidate Short RNA molecules using the method of any of claims 1-4; and
  - (b) testing members of said plurality of candidate Short RNA molecules for the ability to modulate gene expression in said biological system.
60. A method of treating or preventing a disease or disorder in an organism suffering therefrom or predisposed thereto, comprising:
  - (a) identifying a target gene in said organism, wherein said target gene cause or stimulate said disease or disorder;
  - (b) preparing one or more RNAi molecules using the method of any of claims 1-4, wherein said RNAi molecules bind to said one or more target nucleic acid sequences; and
  - (c) contacting said organism with one or more of said RNAi molecules,wherein said RNAi molecules down-regulate the expression of said target gene.

61. A method of killing or slowing or preventing the growth of an organism, comprising:
- (a) identifying a target gene in said organism, wherein said target gene is required for biological functioning of, or stimulates growth of, said organism;
  - (b) preparing one or more RNAi molecules using the method of any of claims 1-4, wherein said RNAi molecules bind to said one or more target nucleic acid sequences; and
  - (c) contacting said organism with one or more of said RNAi molecules,
- wherein said RNAi molecules down-regulate the expression of said target gene, thereby killing or slowing or preventing the growth of said organism.
62. The method of claim 61, wherein said organism is a pathogen.

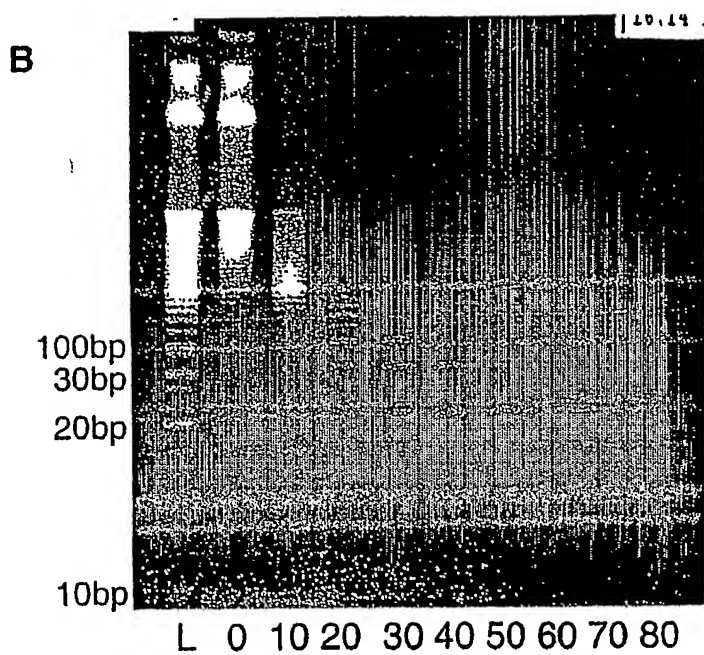
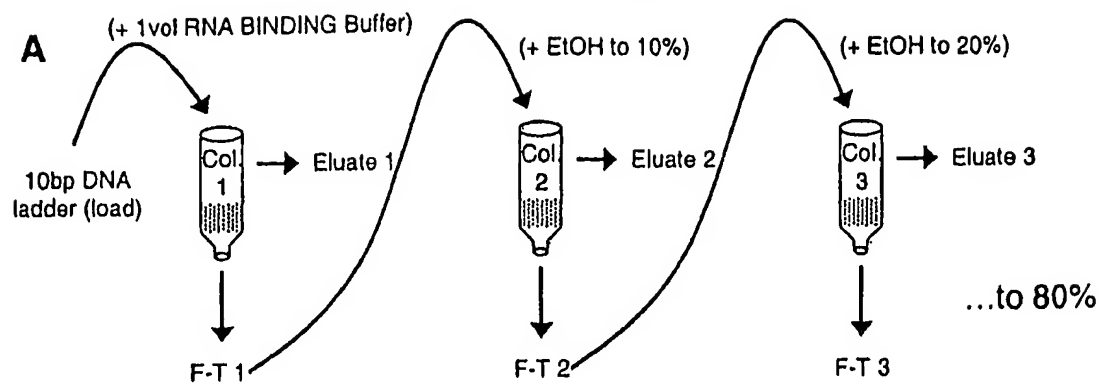
**A****B****Figure 1**



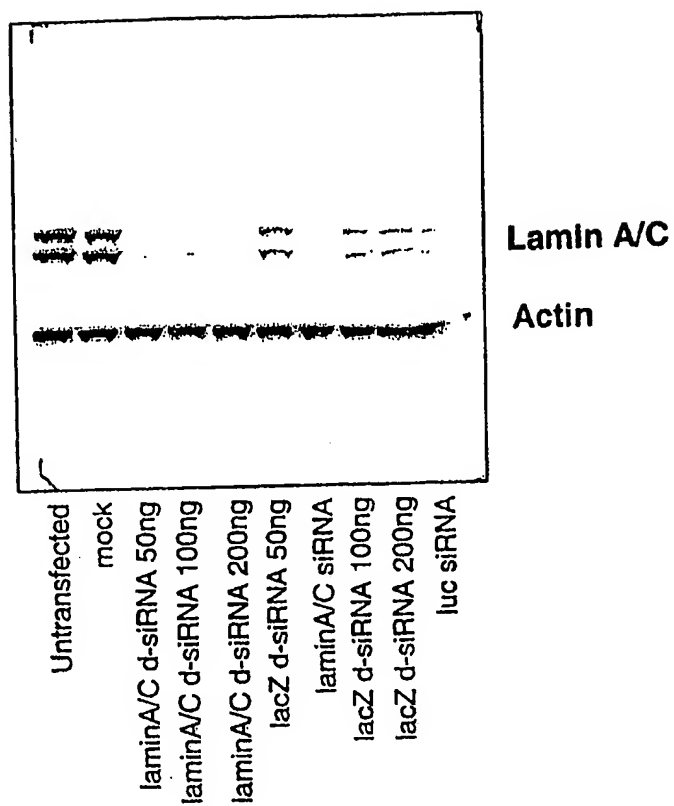
**A****B****Figure 2**



**Figure 3**



**Figure 4**



**Figure 5**

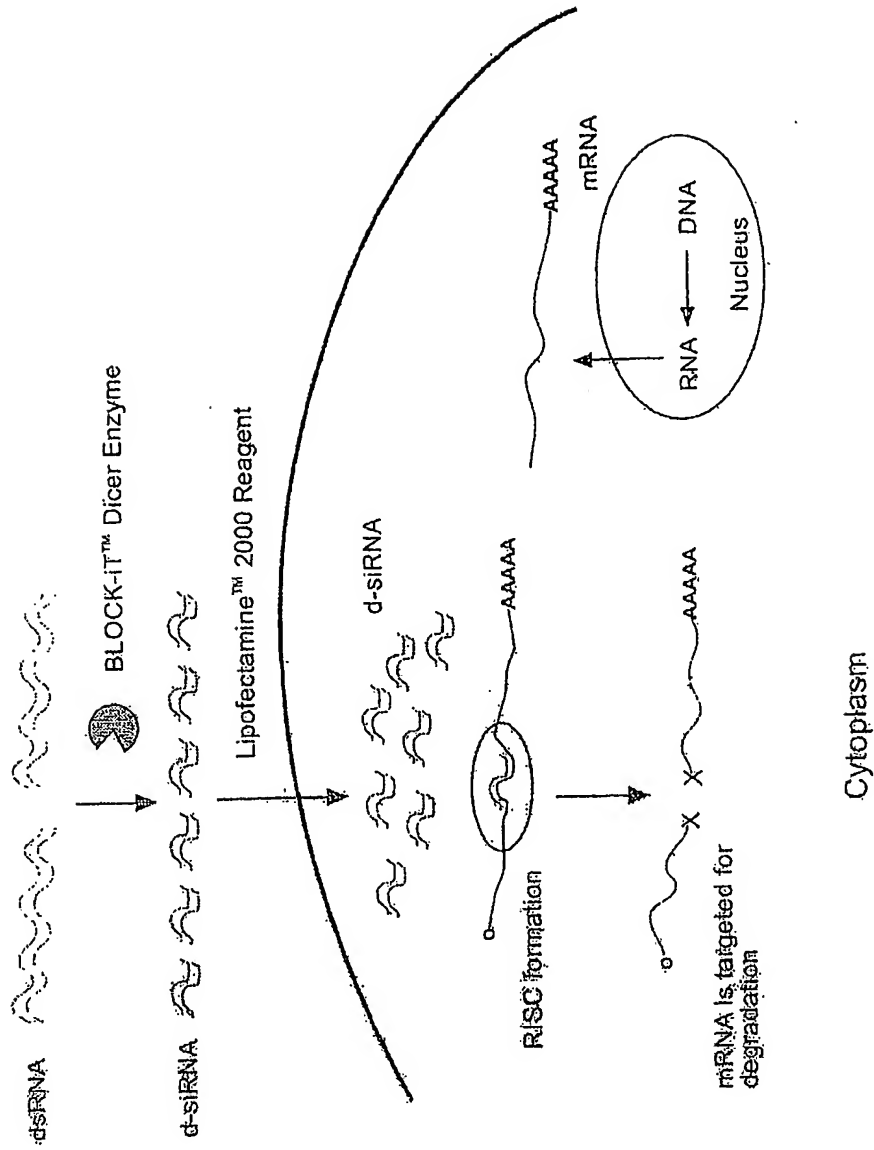


FIG. 6

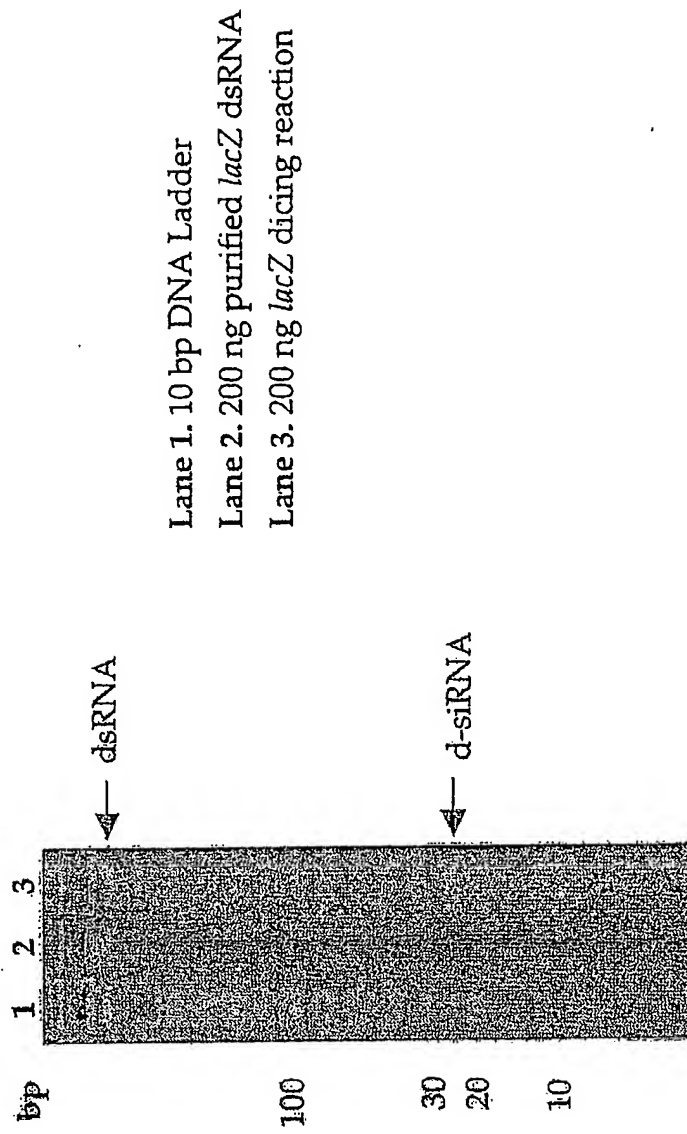


FIG. 7

**Add RNA Binding Buffer and isopropanol to the dicing reaction and mix thoroughly**



FIG. 8

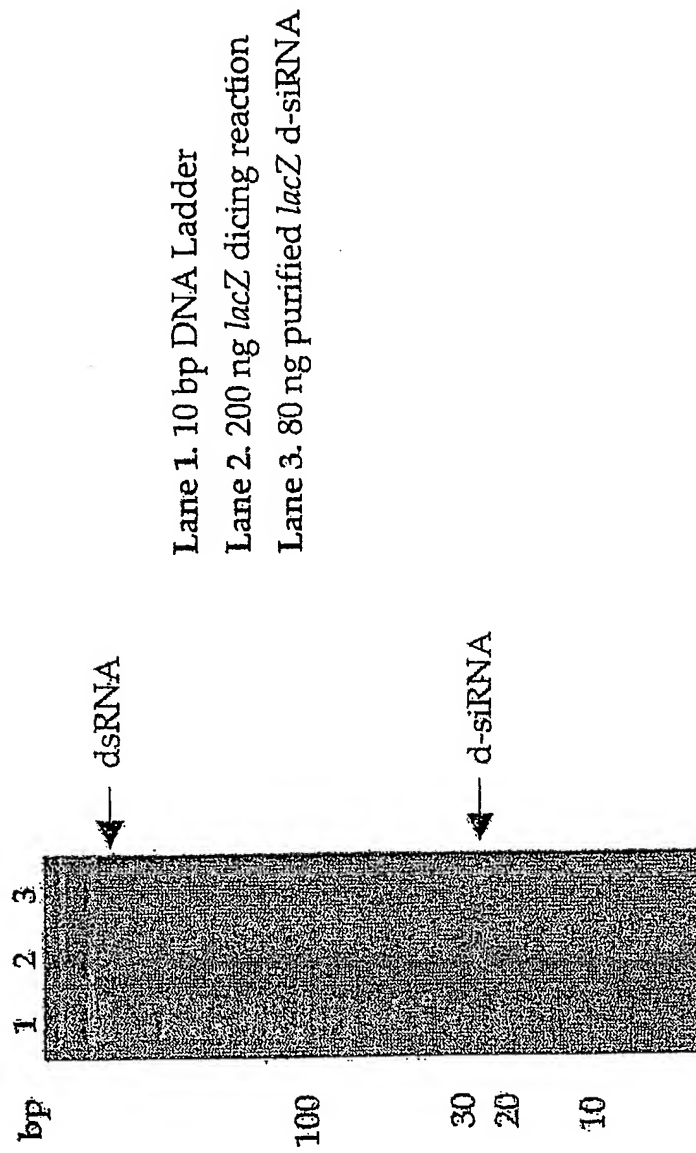


FIG. 9



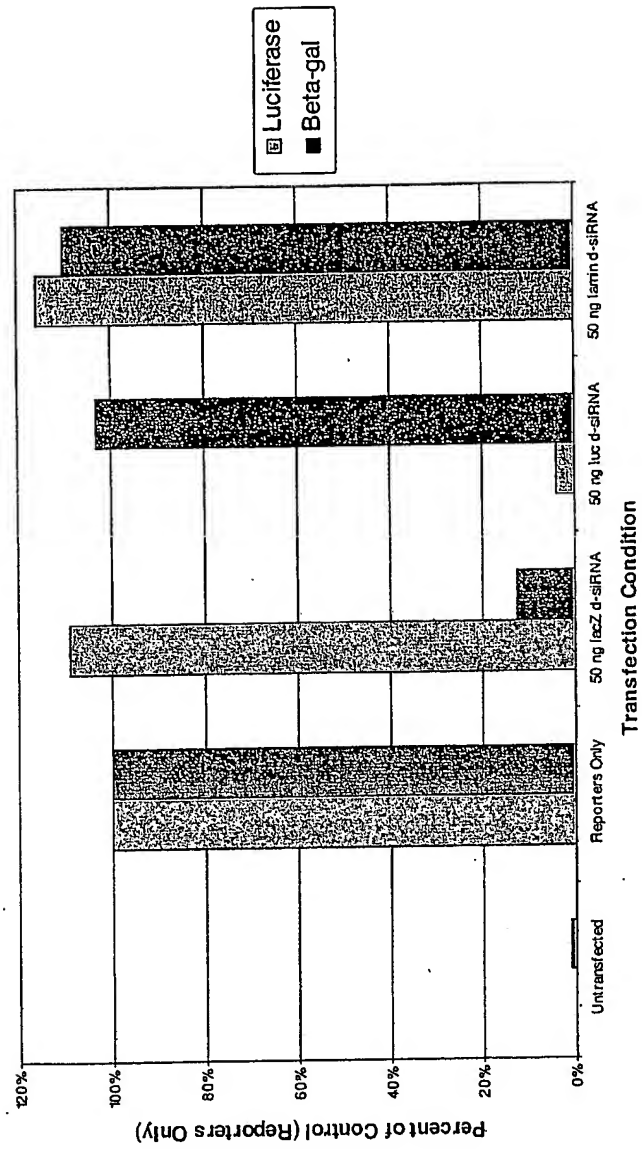


FIG. 10

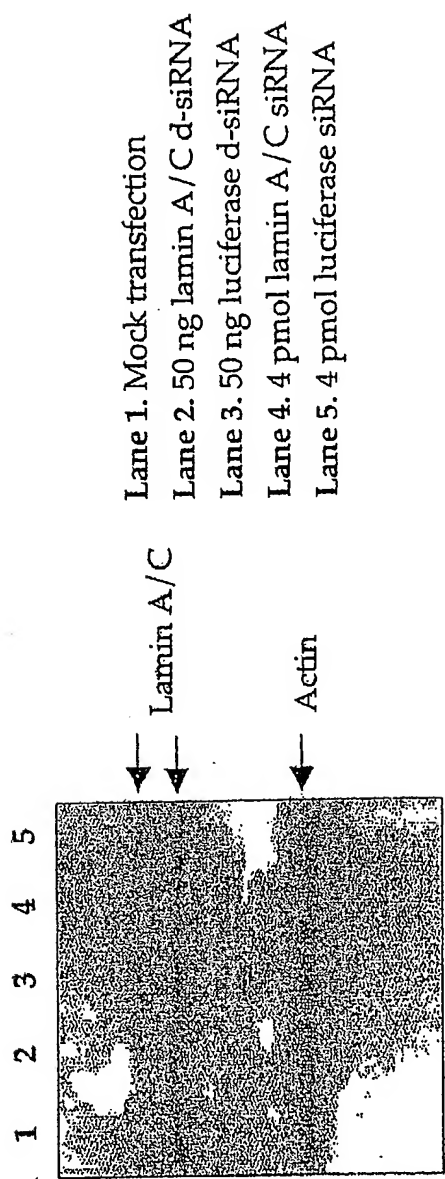


FIG. 11

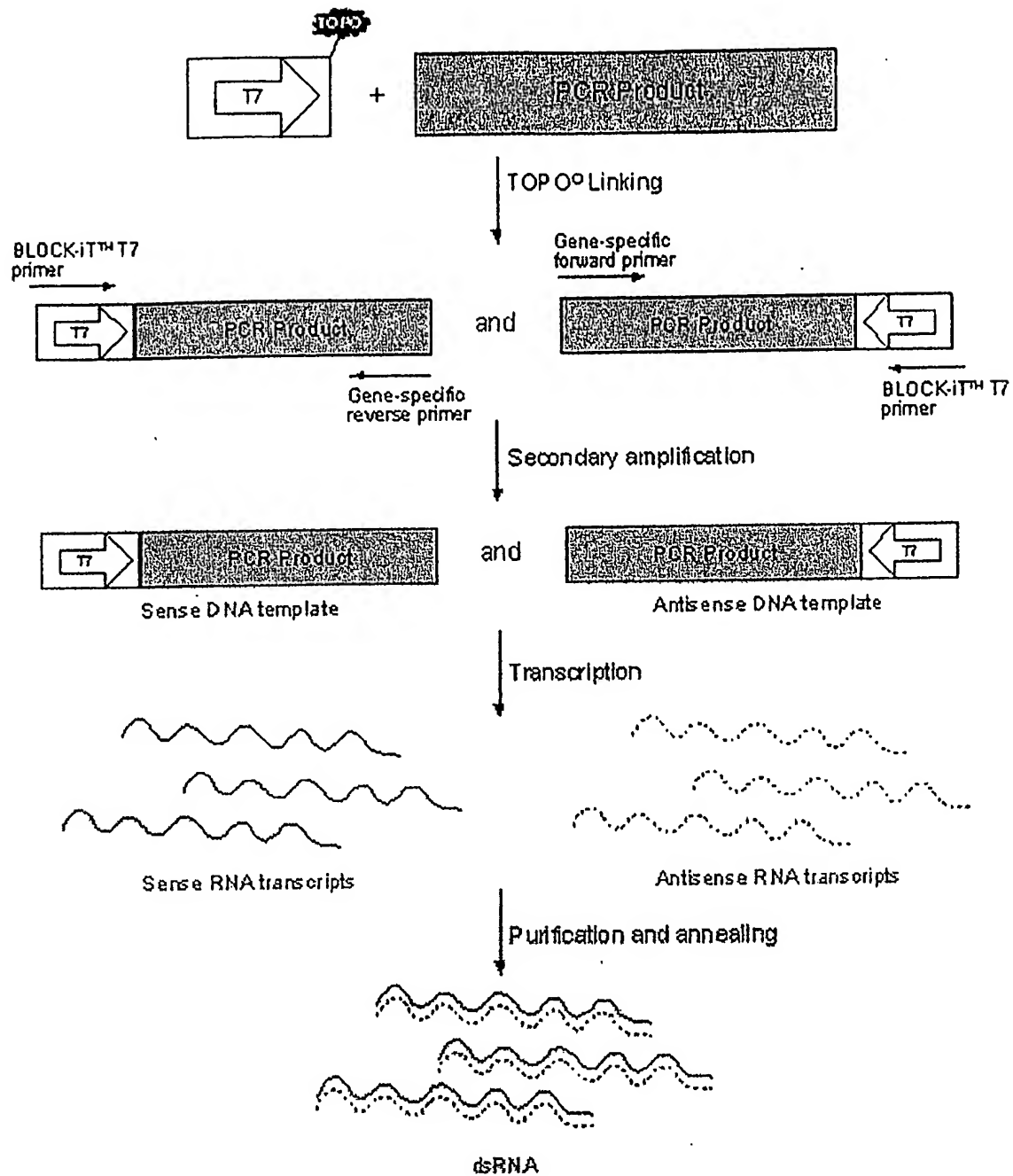


FIG. 12

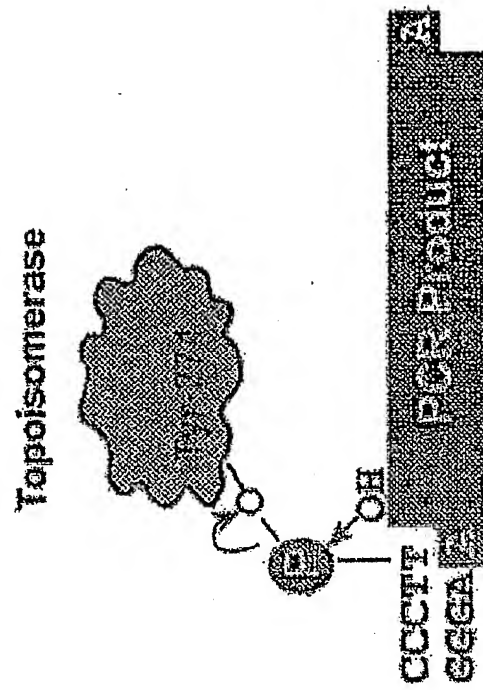


FIG. 13

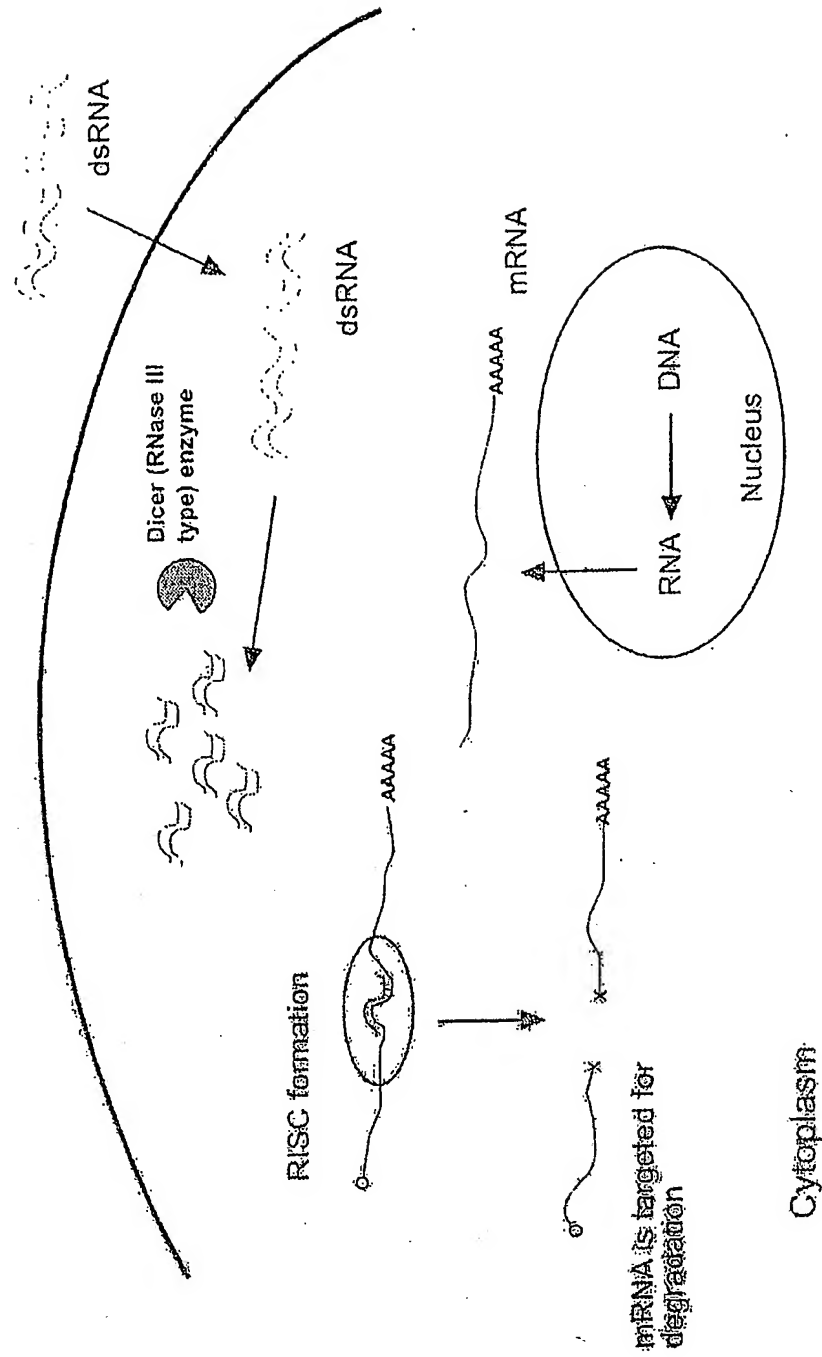


FIG. 14

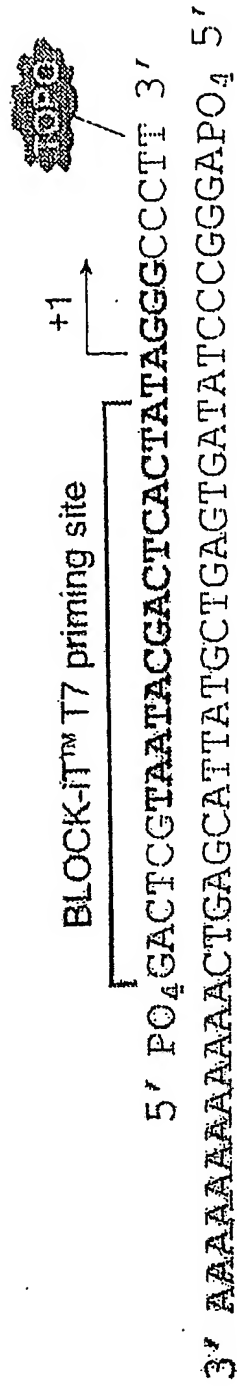
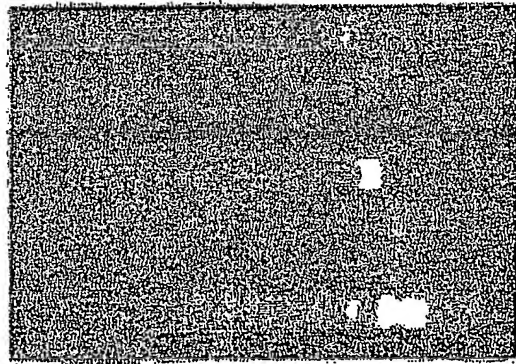


FIG. 15

1 2 3 4 5 6 7



Lane 1. 0.16-1.77 kb RNA Ladder (denatured;  
Invitrogen, Catalog no. 15623-010)

Lane 2. GFP sense transcript

Lane 3. GFP antisense transcript

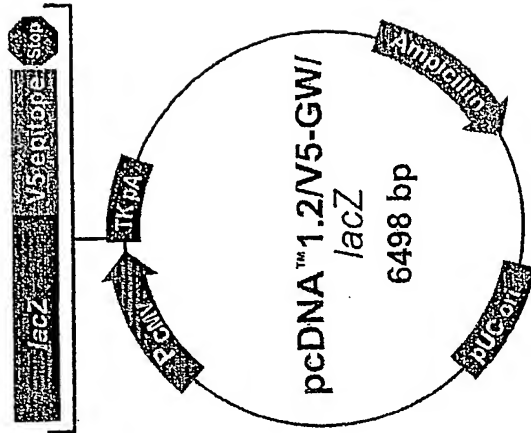
Lane 4. GFP annealed dsRNA

Lane 5. Luciferase sense transcript

Lane 6. Luciferase antisense transcript

Lane 7. Luciferase annealed dsRNA

**FIG. 16**



**Comments for pcDNA™ 1.2/V5-GW/lacZ  
6498 nucleotides**

attB4: bases 5-25  
 CMV promoter: bases 137-724  
 attB1: bases 614-637  
 LacZ fusion protein: bases 643-3798  
 LacZ ORF: bases 643-3714  
 attB2: bases 3716-3739  
 V5 epitope: bases 3739-3780  
 lacZ forward 2 priming site: 840-859  
 lacZ reverse 2 priming site: 1820-1839 (C)  
 TK polyadenylation signal: bases 3807-4078  
 attB3: bases 4079-4099  
 bla promoter: bases 4603-4701  
 Ampicillin (bla) resistance gene: bases 4702-5562  
 pUC origin: bases 5707-6380

(C) = complementary strand

**FIG. 17**



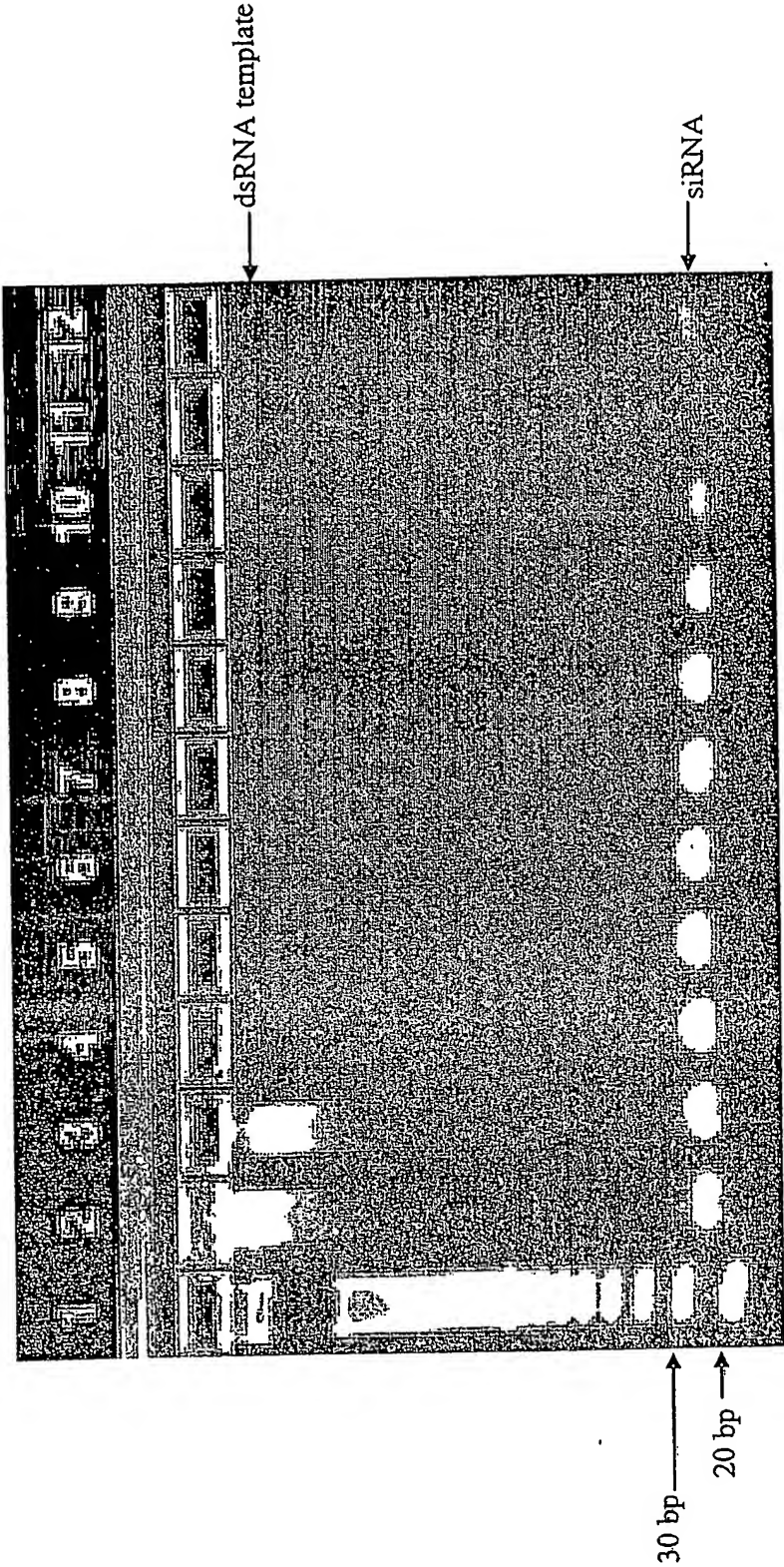


FIG. 18

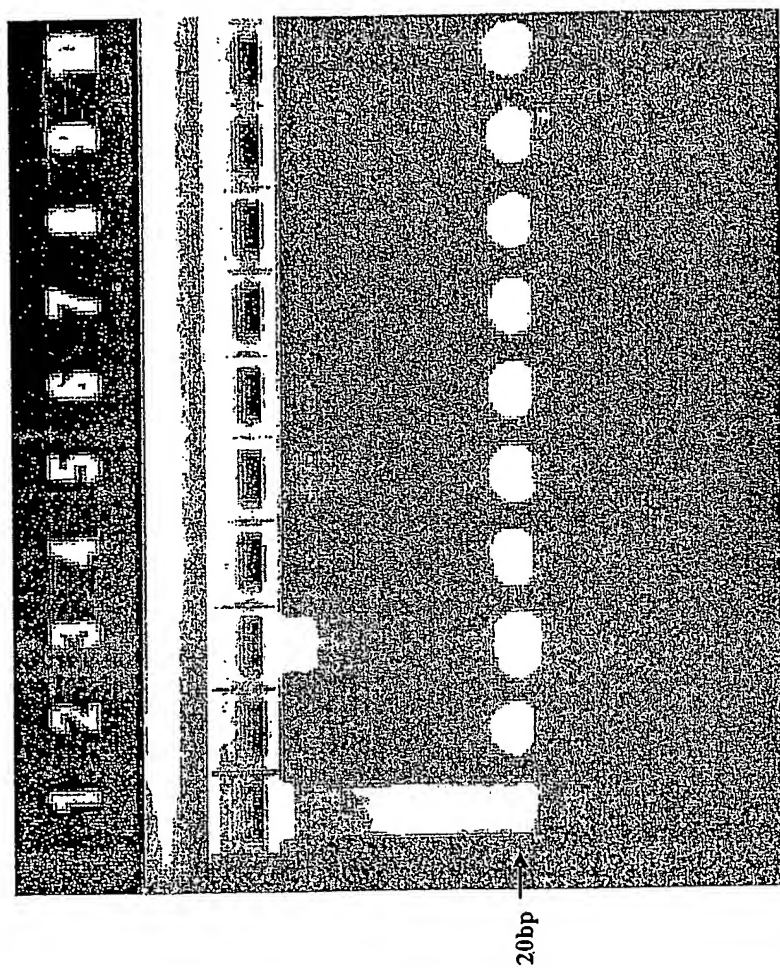


FIG. 19A

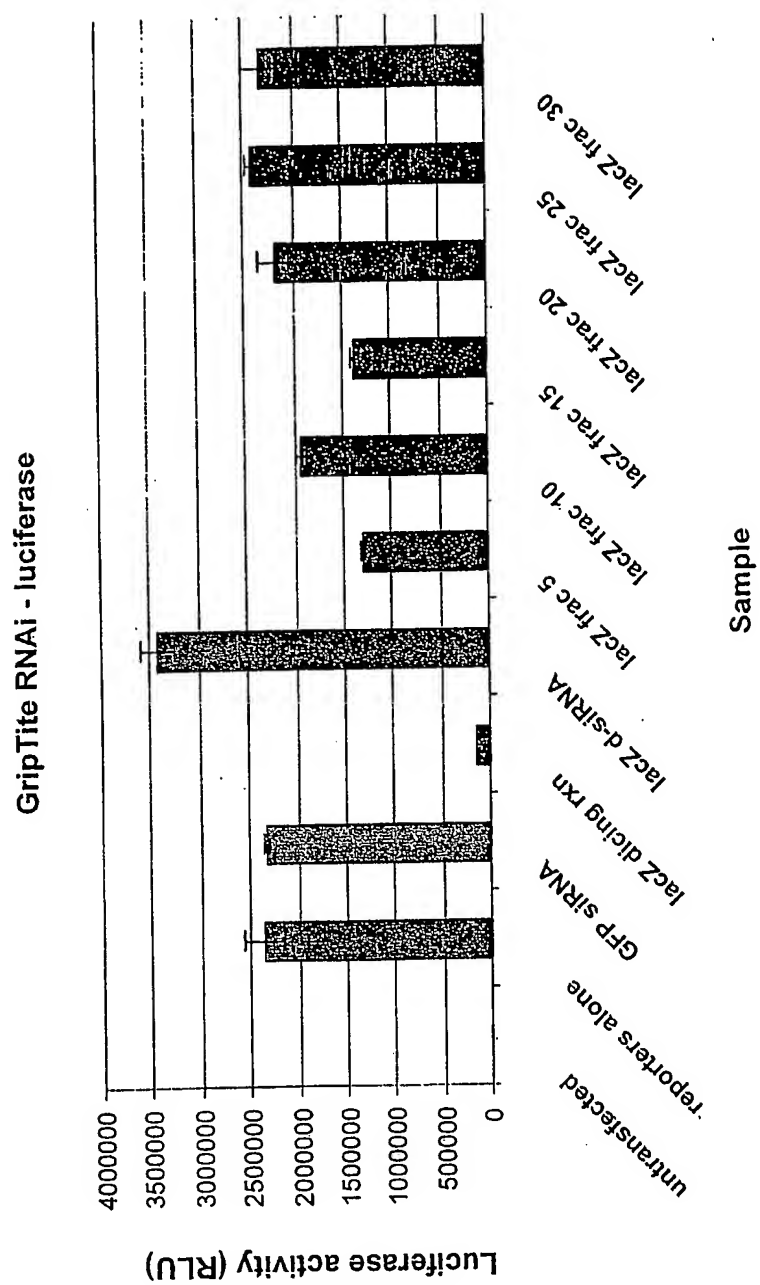


FIG. 19B

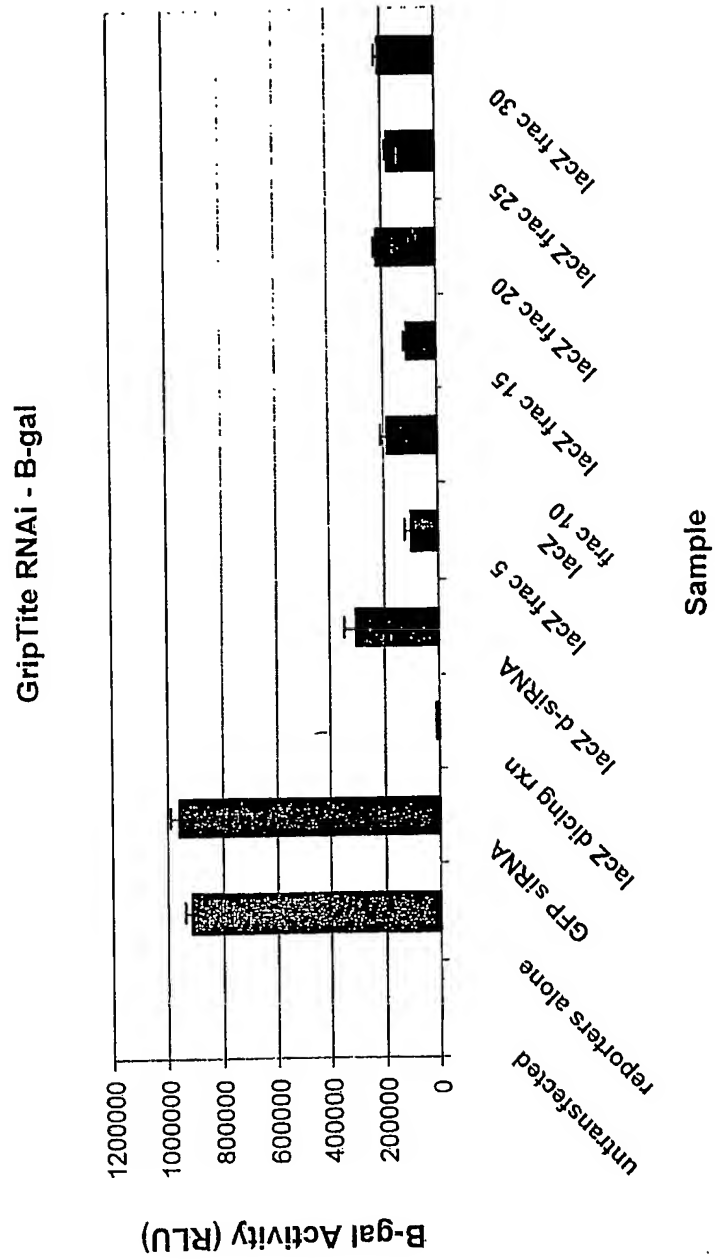


FIG. 19C

Unpurified siRNAs

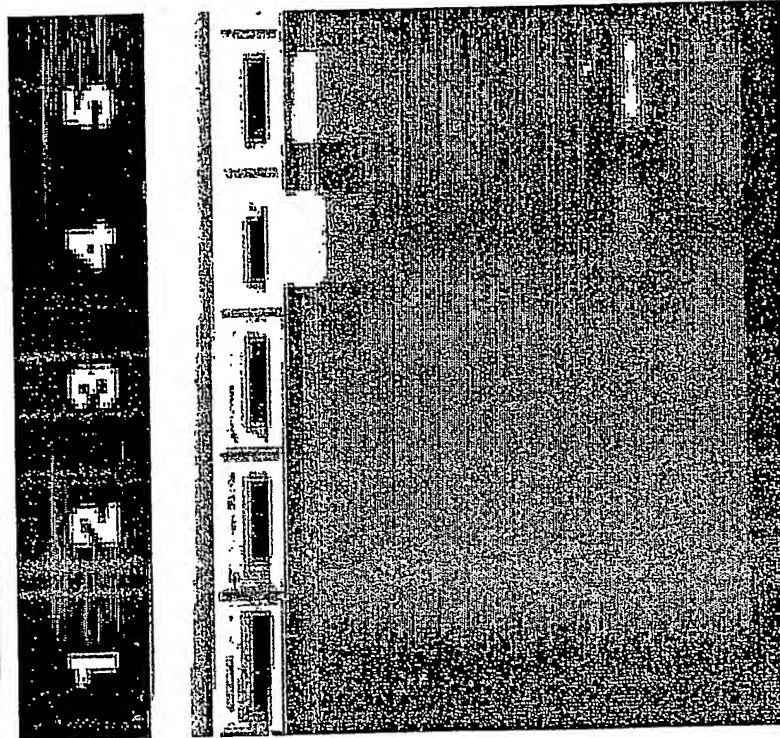


FIG. 20A

Single-Column  
W/ 25% EtOH  
elution

Single-Column  
W/ 30% EtOH  
elution

Two-Column

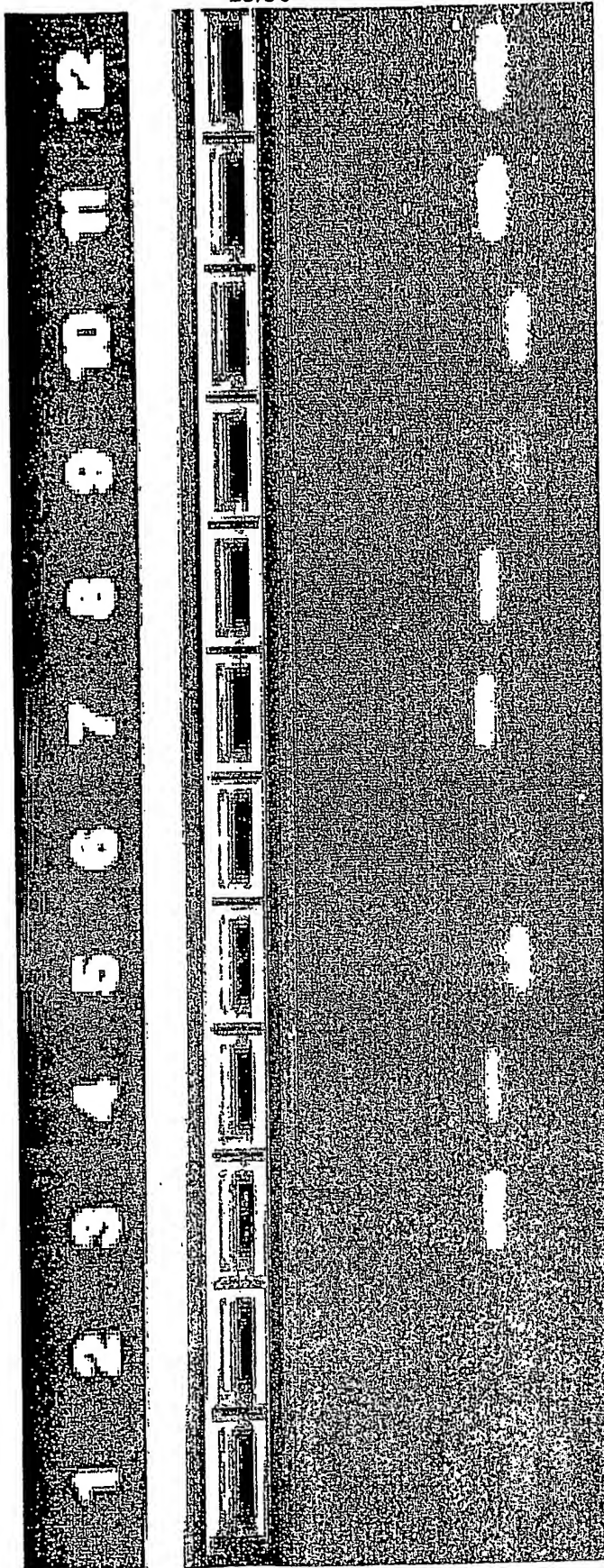


FIG. 20B

FlpIn 293 luc, 1E5 cells/well in 24 well plates

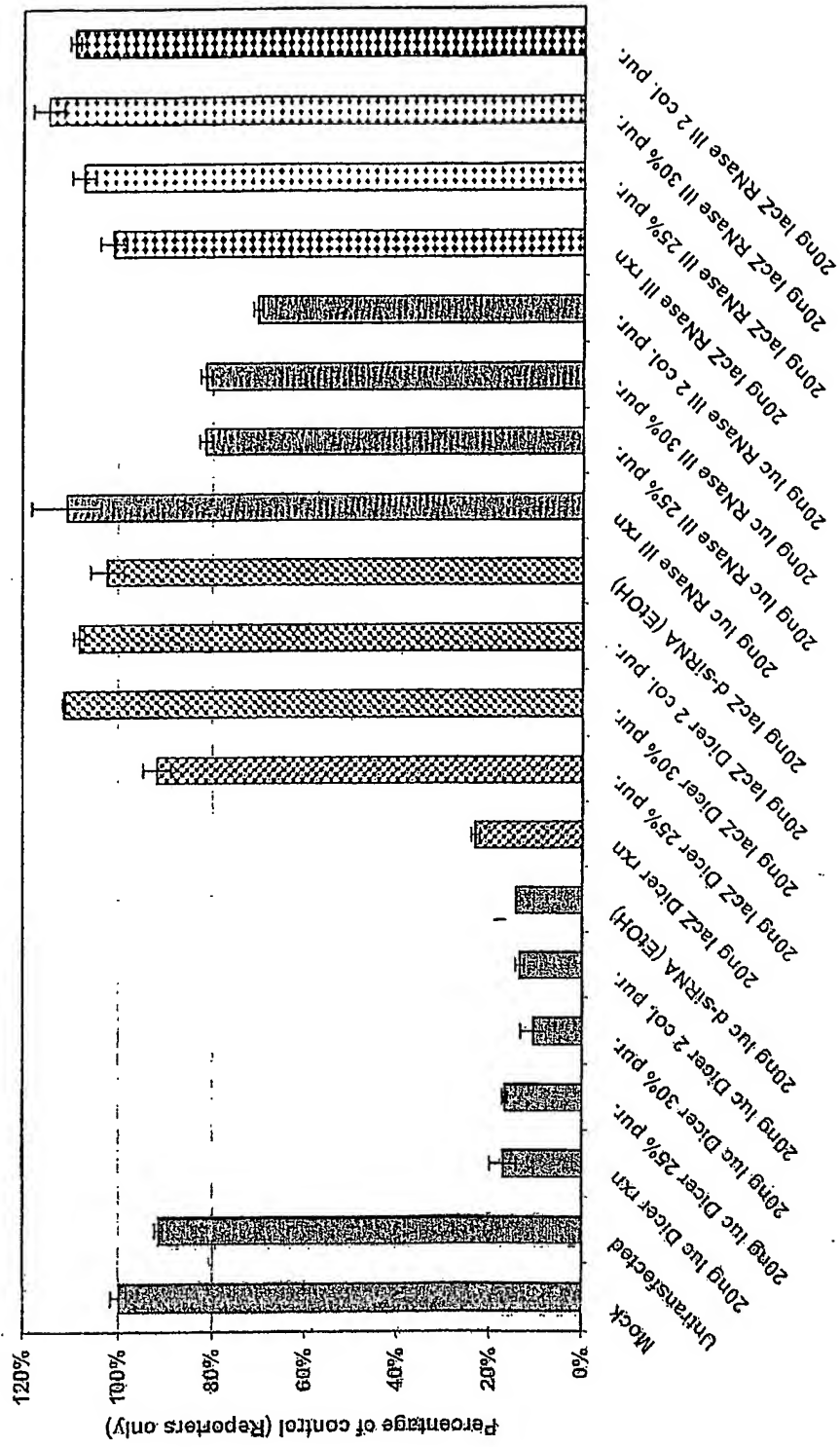


FIG. 21

B-gal Assay  
293 GripTite, 1.5E5 cells/well in 24 well plates

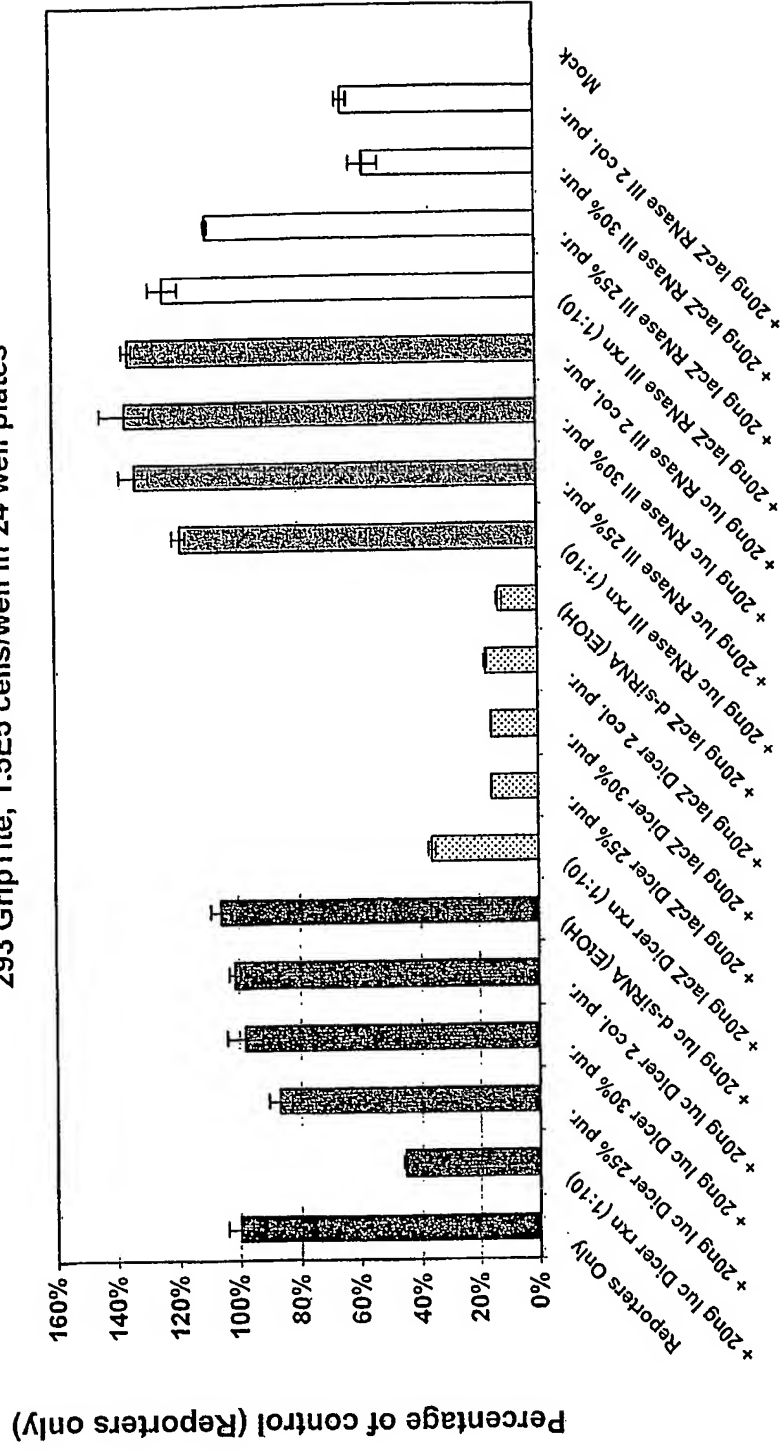


FIG. 22A



Luciferase Assay  
293 GripTite, 1.5E5 cells/well in 24 well plates

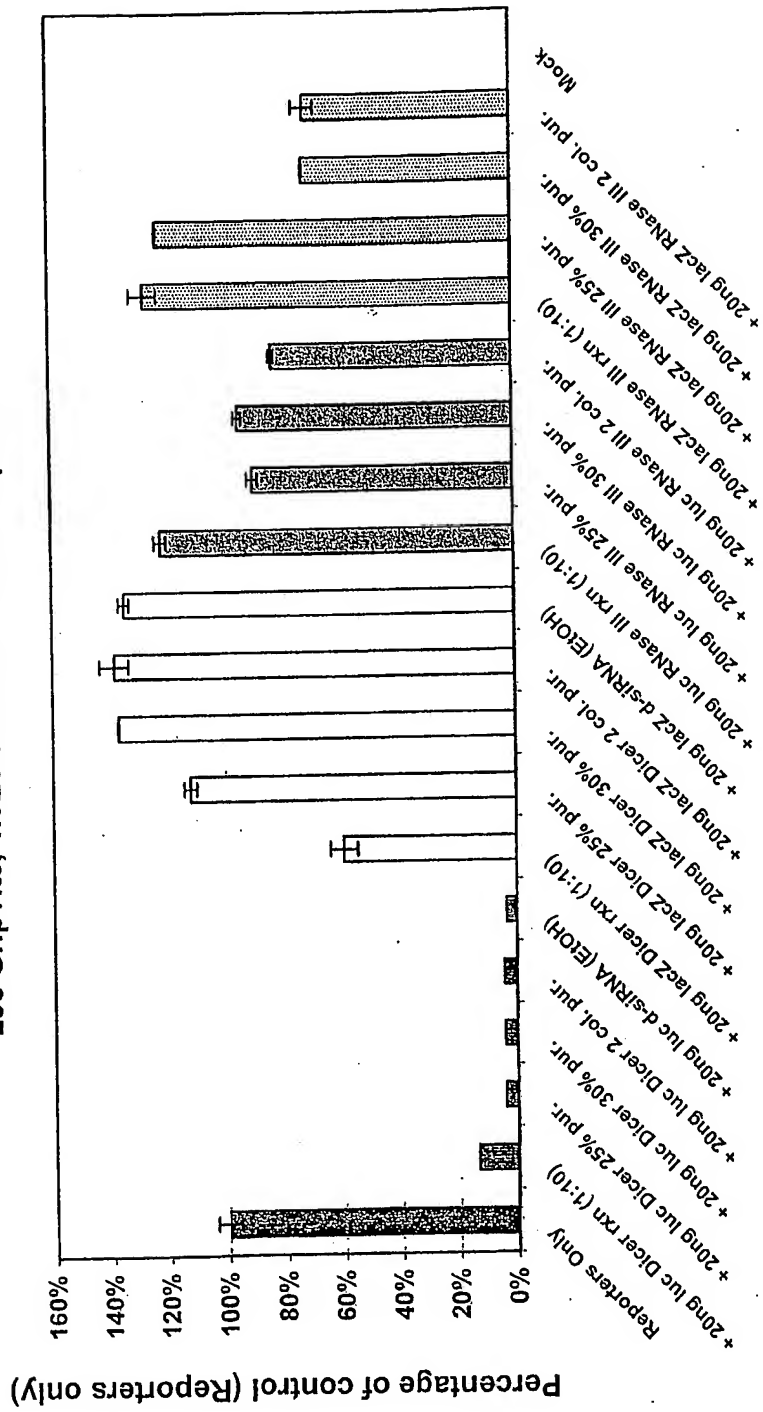
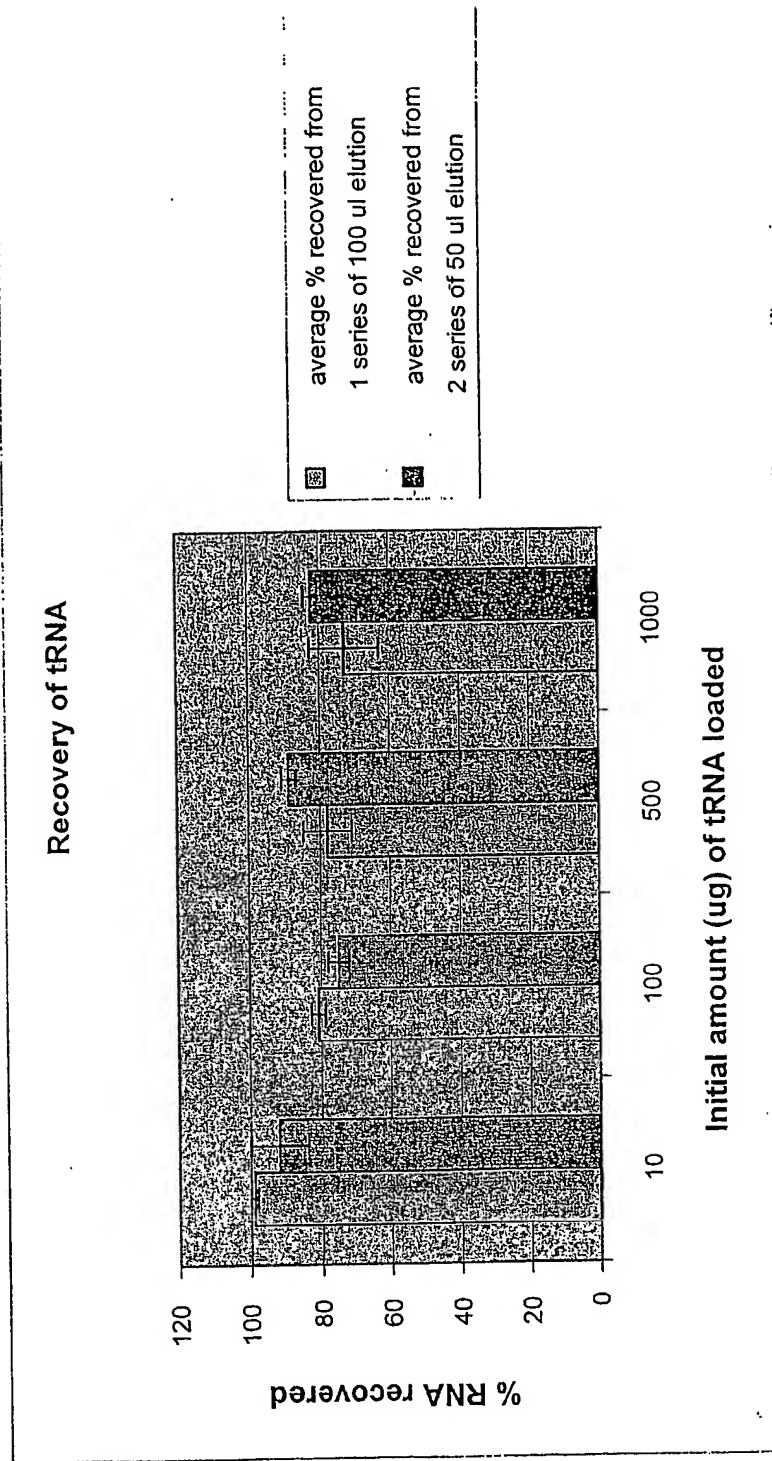


FIG. 22B

**FIG. 23A**

## Recovery of a 1 kb dsRNA Fragment

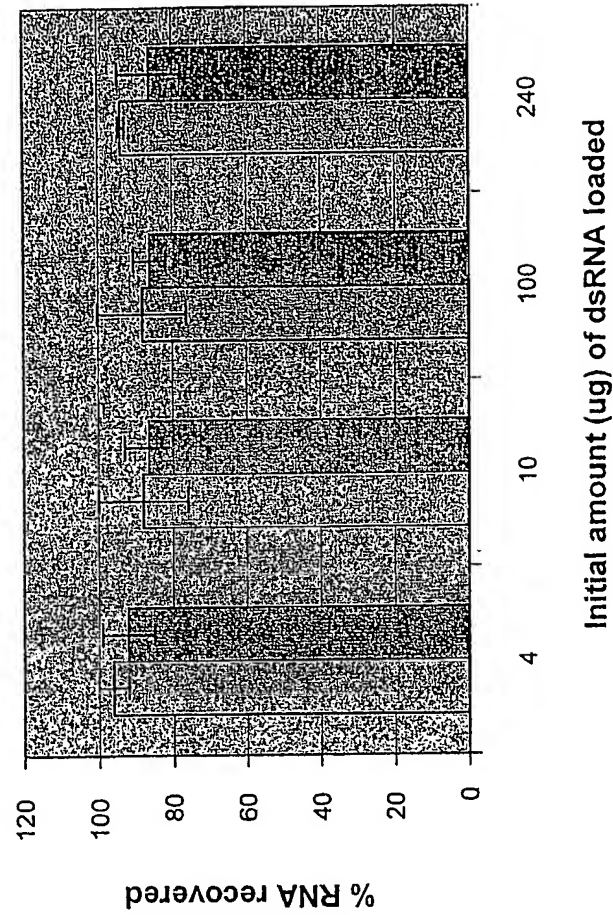


FIG. 23B

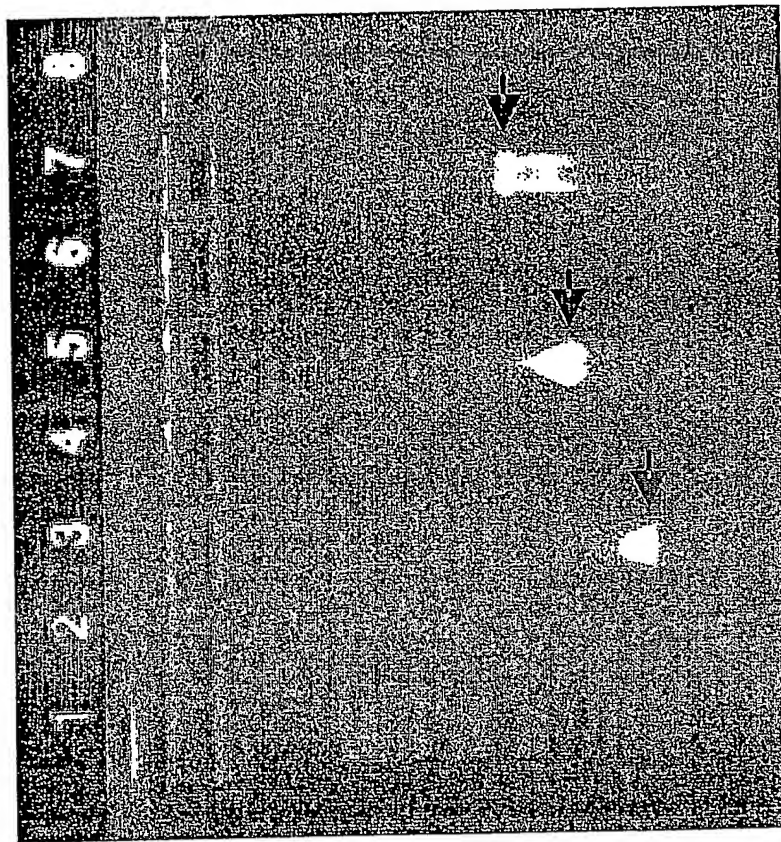


FIG. 24A

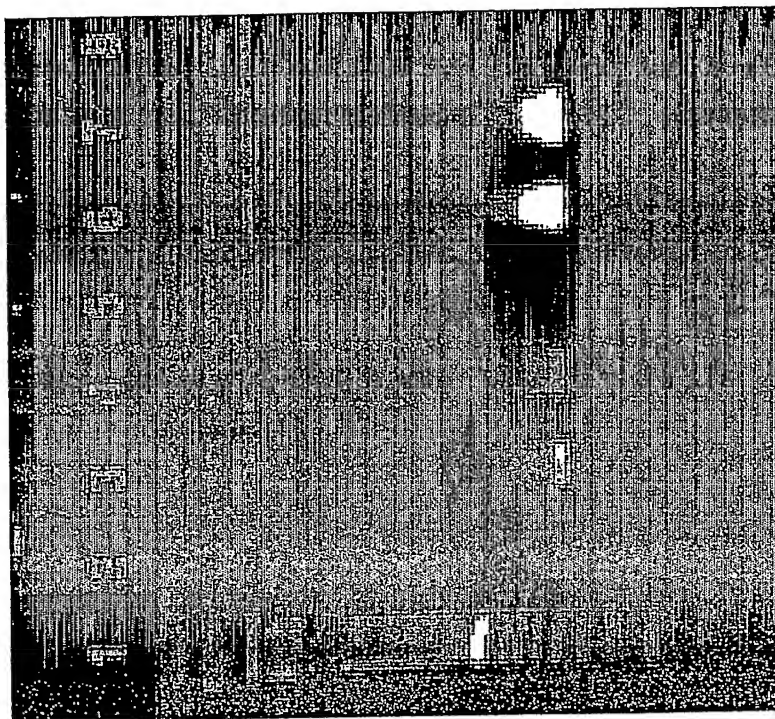


FIG. 24B

## SEQUENCE LISTING

<110> Invitrogen Corporation  
 Madden, Knut R  
 Harris, Adam N  
 Hecker, Karl H  
 Lee, Byung-in

<120> Composition and Methods for Preparing Short RNA Molecules and  
 Other Nucleic Acids

<130> 0942.563PC02

<150> 60/491,758  
 <151> 2003-08-01

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